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**Effects of Artificial Gravity: Central Nervous
System Neurochemical Studies**

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Effects of Artificial Gravity: Central Nervous System Neurochemical Studies

Overview of Project

The major objective of this project was to assess chemical and morphological modifications occurring in muscle receptors and the central nervous system of animals subjected to altered gravity (2 X Earth gravity produced by centrifugation and simulated micro gravity produced by hindlimb suspension). The underlying hypothesis for the studies was that afferent (sensory) information sent to the central nervous system by muscle receptors would be changed in conditions of altered gravity and that these changes, in turn, would instigate a process of adaptation involving altered chemical activity of neurons and glial cells of the projection areas of the cerebral cortex that are related to inputs from those muscle receptors (e.g., cells in the limb projection areas).

The central objective of this research was to expand understanding of how chronic exposure to altered gravity, through effects on the vestibular system, influences neuromuscular systems that control posture and gait. The project used an approach in which molecular changes in the neuromuscular system were related to the development of effective motor control by characterizing neurochemical changes in sensory and motor systems and relating those changes to motor behavior as animals adapted to altered gravity. Thus, the objective was to identify changes in central and peripheral neuromuscular mechanisms that are associated with the reestablishment of motor control which is disrupted by chronic exposure to altered gravity.

Summary of the Research

Effects of Simulated Micro-gravity

Micro-gravity was simulated using the tail suspension method. Specific details of this method and its application for this research are in D'Amelio *et al.*, 1996. A principle objective of this experiment was to evaluate, quantitatively, γ -aminobutyric acid immunoreactivity (GABA-IR) in the hindlimb representation of the rat somatosensory cortex after

14 days of hindlimb unloading. This study was focused on GABAergic neurons since numerous lines of research have demonstrated modifications in the level of GABA-IR or glutamic acid decarboxylase (GAD) immunoreactivity in cortical interneurons when sensory activity is altered by surgical manipulation.

Fixation and Sectioning. After 14 days of tail suspension the animals and their controls were deeply anesthetized with Metophane® and immediately perfused via the heart with 50 ml 0.9% saline, followed by 500 ml of a fixative made up of 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed the same day, immersed in fresh fixative and stored at 4°C.

The right hemisphere was coronally blocked between Bregma -1.8mm and Bregma -3.6mm where the somatosensory representation of the hindlimb is conspicuous and associated with the presence of the rostral hippocampus (Paxinos and Watson, 1986). At this level the more rostrally located forelimb representation is no longer present (rostral to Bregma -1.8 the somatosensory cortex contains both hindlimb and the laterally adjacent forelimb representations. The hippocampus is not visible). Forty µm-thick coronal sections were cut on a Vibratome® and collected in TBS (0.05 M Tris buffer-0.9% saline, pH 7.6). Twenty serial sections per animal were used for the staining procedures; 15 were stained for immunocytochemistry and 5 were Nissl-stained with cresyl violet to identify the cytoarchitectonic layers of the hindlimb representation.

GABA Immunocytochemistry. Floating sections were first incubated for 5-10 min at room temperature (RT) with 3% hydrogen peroxide in 10% methanol in TBS and subsequently rinsed 4 times in TBS x 30 min (RT). The sections were then immersed in GABA antiserum (Chemicon, Cat.# AB131) or control serum (preimmune rabbit serum) diluted at 1:1000 in TBS for 48-72 h at 4°C, with orbital agitation. Then, they were rinsed 4 times in TBS x 30 min (RT) and incubated for 60 min (RT) in swine anti-rabbit IgG diluted 1:50 in TBS. The sections were rinsed 4 more times in TBS x 30 min (RT) and then incubated for 60 min (RT) with rabbit peroxidase-antiperoxidase complex (Sigma) diluted 1:200 in TBS. To develop reaction product the sections were immersed in 12.5 mg diaminobenzidine tetrahydrochloride in 50 ml TBS + 5 µl 30% hydrogen peroxide for 5-8 min. Finally, they were rinsed in TBS, 2 changes x 10 min

(RT), mounted on gelatin coated slides, air-dried and coverslipped with Permount®.

The sections from pairs of experimental and control animals were processed together in the same solutions for consistent immunostaining. For identification purposes, the hemisphere of the control rat was marked with a small hole at the level of the striatum. Sections of each suspended and control pair were placed on the same glass slide for counting of GABA-IR cells.

Methodology for Quantitative Analysis. The hindlimb somatosensory cortex was identified in Nissl-stained slides by the prominent aggregation of granular cells in layer IV. The boundaries of the hindlimb representation were drawn on a piece of white paper. The projected image of the sections stained with GABA antiserum was superimposed on the drawing and GABA-IR cells intensely or moderately stained were marked on the paper. Blood vessels as well as meningeal foldings served as reference marks for each section. The marking of the cells slightly exceeded the lateral and medial boundaries of the hindlimb representation. Subsequently, the coverslips of the anti-GABA stained slides were removed by soaking in xylene and the sections were Nissl-stained with cresyl violet and remounted. The Nissl-staining of the slides in which the counting of the GABA-IR cells was previously made, gave us more confidence in tracing the boundaries of the area and demarcating the cortical laminae based on the prominent granular aggregates of layer IV. The projected image of these sections was drawn on a translucent sheet of paper. The drawing included the boundaries of the hindlimb representation, the reference marks and the dividing lines of six cortical layers identified as layer I, II/III, IV, Va, Vb and VI (see Zilles and Wree, 1985). This drawing was then overlaid on the paper that had the markings of GABA-IR cells. The boundaries of the hindlimb cortex were then corrected and GABA-IR cells were counted in each layer on the translucent paper (See figs. 1 and 2)

The image of each layer on this translucent sheet was captured into a Macintosh Centris 650 computer using a Sierra Scientific Model MS4030 CCD tube camera that had a macro Nikon/Nikor 55 lens and a Scion Technology LG-3 frame grabber board in the Nubus slot of the computer. An image of standard square inches etched in the copy stand was also

captured and then used to compute the correction factor for the distortion of the aspect ratio introduced by the camera lens and the computer monitor. Quantitative measurements of the cortical layers were done blind by one of us (L.C.W.). The digitized images were magnified at 2x, and a sharpening filter was used prior to measuring. Measurements are based on four to eight GABA/Nissl-stained slides for each of the three rats in each group.

Results and Conclusions. A reduction in number of GABA-immunoreactive cells with respect to the control animals was observed in layer Va and Vb. GABA-containing terminals were also reduced in the same layers, particularly those terminals surrounding the soma and apical dendrites of pyramidal cells in layer Vb. On the basis of previous morphological and behavioral studies of the neuromuscular system of hindlimb-suspended animals, it was suggested that the unloading due to hindlimb suspension alters afferent signalling and feedback information from intramuscular receptors to the cerebral cortex due to modifications in the reflex organization of hindlimb muscle groups. We proposed that the reduction in immunoreactivity of local circuit GABAergic neurons and terminals is an expression of changes in their modulatory activity to compensate for the alterations in the afferent information.

Development of Method for Quantifying GABA-IR

A computer-based method for the quantitative assessment of the area occupied by immunoreactive terminals in close apposition to nerve cells in relation to the perimeter of the cell soma was developed to facilitate analysis of GABA-IR. This method is based on Fast Fourier Transform (FFT) routines incorporated in NIH-Image public domain software. Pyramidal cells of layer V of the somatosensory cortex outlined by GABA immunolabeled terminals were chosen for our analysis. A Leitz Diaplan light microscope was employed for the visualization of the sections. A Sierra Scientific Model 4030 CCD camera was used to capture the images into a Macintosh Centris 650 computer. After preprocessing, filtering was performed on the power spectrum in the frequency domain produced by the FFT operation. An inverse FFT with filter procedure was employed to restore the images to the spatial domain. Pasting of the original image to the transformed one using a Boolean logic operation called "AND"ing

produced an image with the terminals enhanced. This procedure allowed the creation of a binary image using a well-defined threshold of 128. Thus, the terminal area appears in black against a white background. This methodology provides an objective means of measurement of area by counting the total number of pixels occupied by immunoreactive terminals in light microscopic sections in which the difficulties of labeling intensity, size, shape and numerical density of terminals are avoided.

Effects of Hyper-Gravity

Quantitative evaluation of GABA-IR in the hindlimb representation of the rat somatosensory cortex after 14 days of exposure to hypergravity (hyper-G). The computer-assisted image procedure described in the foregoing was employed in this investigation. The methodology for fixation and sectioning of the tissue and for immunocytochemical staining used the procedure applied in the hindlimb suspension study.

Results and Conclusions. The area of GABA-IR axosomatic terminals apposed to pyramidal cells of cortical layer V was reduced in rats exposed to hyper-G as compared with control rats which were exposed either to rotation alone or to vivarium conditions (see Table I). Thus, chronic exposure to either simulated micro-gravity and hyper-gravity produced by centrifugation elicited changes in GABA-IR in areas of the sensory motor cortex which receive projections from muscle afferents.

Table 1. Average ratio of terminal area to perimeter of the soma for each of the 11 rats used in the experiment. Data are presented for staining triplets of three rats where tissue of centrifuged (3G), vivarium (VIV) and rotation (RC) control rats were immunostained concurrently and mounted on single slides. Numbers in parentheses identify the number of slides and the number of cells (slides; cells) contributing to each mean for each rat .

We believe that the reduction observed in GABA-IR of the terminal area around pyramidal neurons reveals that inhibitory influences in the central nervous system respond to adjust central motor control programs in conditions of non-invasive manipulations, i.e., altered gravity. On the basis of behavioral studies of the neuromuscular system of centrifuged animals, we believe that the modifications in muscle activity occurring during exposure to hyper-G alters the afferent input and feedback

information from muscle receptors which in turn affects the processing of information in areas of the cerebral cortex related to the proprioceptive input from muscle groups. As a consequence, priorities for muscle recruitment are altered at the cortical level. We believe the changes in GABA-IR that occur following chronic exposure to altered gravity reflect changes in CNS neurotransmitter systems that are involved in adaptation of the neuromuscular system to new environmental conditions. Because GABA-IR is altered from chronic exposure to either simulated micro-gravity and hyper-gravity, we believe the GABAergic system is importantly involved in as a "basic" adaptive mechanism in motor control.

<i>Staining triplets</i>	3G	VIV	RC
Group 1	6.78 (3; 19)	9.56 (3; 18)	8.96 (3; 17)
Group 2	5.54 (3; 23)	6.37 (3; 24)	4.88 (2; 16)
Group 3	5.27 (5; 40)	9.41 (5; 39)	8.94 (5; 40)
Group 4	4.95 (3; 17)	6.16 (3; 13)	X
Mean	5.63	7.88	7.59
SD	0.80	1.86	2.35
SEM	0.40	0.93	1.36
t test vs VIV	3.37	- -	0.25
p value	<.05	- -	>.20

Publications and Professional Activity

The following publications and presentations were supported in part on their entirety by funding for this project.

Papers and Chapters

- D'Amelio, F., Wu, L.C., Fox, R.A., Daunton, N.G., Corcoran, M.L. & Polyakov, I. (1997) Hypergravity exposure decreases GABA immunoreactivity in axon terminals contacting pyramidal cells in the rat somatosensory cortex: a quantitative immunocytochemical image analysis. (submitted to the Journal of Comparative Neurology).
- D'Amelio, F., Fox, R.A., Wu, L.C., Daunton, N.G., & Corcoran, M.L. (1997) Effects of microgravity on muscle and cerebral cortex: a suggested interaction. Advances in Space Research (in press).
- Wu, L.C., D'Amelio, F., Fox, R.A., Polyakov, I. and Daunton, N.G. (1997) Light microscopic image analysis system to quantify immunoreactive terminal area apposed to nerve cells. Journal of Neuroscience Methods, 74: 89-96, 1997.
- D'Amelio, R., Fox, R., Wu, L.C., Daunton, N. (1996). Quantitative changes of GABA-immunoreactivity in the hindlimb representation of the rat somatosensory cortex after 14-day hindlimb unloading by tail suspension. Journal of Neuroscience Research, 44, 532-539.
- Meza, G., Bohne, B., Daunton, N., Fox, R., and Knox, J. (1996). Recovery of otolithic function following streptomycin treatment in the rat. In New Directions in Vestibular Research. New York: New York Academy of Sciences.
- Sergutina, A., Gershtein, L., D'Amelio, F., Daunton, N., Krasnov, I. (1995). Some cytochemical features of the motor system of the rat brain after space flight. Byulleten Eksperimental'noi Biologii i Meditsini [Bulletin of Experimental Biology and Medicine], Russia, 119(3), 288-290.
- Fox, R., Corcoran, M., Daunton, N., and Morey-Holton, E. (1994). Effects of spaceflight and hindlimb suspension on the posture and gait of rats. In: Taguchi, K., Igarashi, M., and Mori, W. (Eds) Vestibular and Neural Front. Amsterdam: Elsevier Science B. V., pp. 603-606.

Published Abstracts

- D'Amelio, F., Fox, R., Wu, L.C., Daunton, N. (1995). Quantitative changes of GABA-immunoreactivity in the hindlimb representation of the rat somatosensory cortex after 14-day hindlimb unloading by tail suspension. Neuroscience Abstracts, 21, 1901.
- Daunton, N., Corcoran, M., Fox, R., Wu, L.C., D'Amelio, F., and Polyakov, I. (1995). Behavioral studies on recovery of vestibular function following chronic exposure to different levels of hyper gravity. ASGSB Bulletin, 9(1), 66.

Fox, R.A., Knox, J., Skinner, J., & Spomer, M. (1995). Functional deafferentation of knee joint afferents produces leg extension and knuckle walking in rats. Neuroscience Abstracts, 21, 240.

Polyakov, I., D'Amelio, F., Daunton, N., Fox, R., Corcoran, M., and Wu, L-C. (1995). Preliminary studies on the effects of artificial gravity: Immunocytochemical findings in areas of the central nervous system involved in motor behavior. ASGSB Bulletin, 9(1), 40.

Sergutina, A., Gershtein, L., D'Amelio, F., Daunton, N., Krasnov, I. (1994). Cytochemical analysis of the somatosensory cortex and caudate nucleus of the rat brain after 9-day space flight. In: X Conference Space Biology and Aviaspace Medicine. Moscow, Russia, 158.

Papers at Scientific Meetings

D'Amelio, F., Fox, R.A., Wu, L.C., Daunton, N.G., & Corcoran, M.L. (1997) Effects of microgravity on muscle and cerebral cortex: a suggested interaction. Meeting of the Committee on Space Research, Birmingham UK, June.

D'Amelio, R., Fox, R., Wu, L-C., Daunton, N. (1995). Quantitative changes of GABA-immunoreactivity in the hindlimb representation of the rat somatosensory cortex after 14-day hindlimb unloading by tail suspension. Meeting of the Society for Neuroscience, San Diego, CA, Nov.

Daunton, N., Corcoran, M., Fox, R., Wu, L-C., D'Amelio, F., and Polyakov, I. (1995). Behavioral studies on recovery of vestibular function following chronic exposure to different levels of hyper gravity.

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Meza, G., Daunton, N., Fox, R., Lopez-Griego, L., and Zepeda, H. (1994). Restoration of vestibular function in streptomycin-treated rats: Behavioral studies. Meeting of the International Society for Developmental Neuroscience. San Diego, August.

Meza, G., Daunton, N., Fox, R., Lopez-Griego, L., and Zepeda, H. (1994). Behavioral studies on recovery of vestibular function in streptomycin-treated rats. Conference on Sensory Generation. Charlottesville, VA, May.

Polyakov, I., D'Amelio, F., Daunton, N., Fox, R., Corcoran, M., and Wu, L-C. (1995). Preliminary studies on the effects of artificial gravity: Immunocytochemical findings in areas of the central nervous system involved in motor behavior.

Sergutina, A., Gershtein, L., D'Amelio, F., Daunton, N., Krasnov, I. (1994). Cytochemical analysis of the somatosensory cortex and caudate nucleus of the rat brain after 9-day space flight. In: X Conference Space Biology and Aviation Space Medicine. Moscow, Russia, 158.

EFFECTS OF MICROGRAVITY ON MUSCLE AND CEREBRAL CORTEX: A SUGGESTED INTERACTION

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ABSTRACT

The "slow" antigravity muscle adductor longus was studied in rats after 14 days of spaceflight (SF). The techniques employed included standard methods for light microscopy, neural cell adhesion molecule (N-CAM) immunocytochemistry and electron microscopy. Light and electron microscopy revealed myofiber atrophy, segmental necrosis and regenerative myofibers. Regenerative myofibers were N-CAM immunoreactive (N-CAM-IR). The neuromuscular junctions showed axon terminals with a decrease or absence of synaptic vesicles, degenerative changes, vacant axonal spaces and changes suggestive of axonal sprouting. No alterations of muscle spindles was seen either by light or electron microscopy. These observations suggest that muscle regeneration and denervation and synaptic remodeling at the level of the neuromuscular junction may take place during spaceflight.

In a separate study, GABA immunoreactivity (GABA-IR) was evaluated at the level of the hindlimb representation of the rat somatosensory cortex after 14 days of hindlimb unloading by tail suspension ("simulated" microgravity). A reduction in number of GABA-immunoreactive cells with respect to the control animals was observed in layer Va and Vb. GABA-IR terminals were also reduced in the same layers, particularly those terminals surrounding the soma and apical dendrites of pyramidal cells in layer Vb. On the basis of previous morphological and behavioral studies of the neuromuscular system after spaceflight and hindlimb suspension it is suggested that after limb unloading there are alterations of afferent signaling and feedback information from intramuscular receptors to the cerebral cortex due to modifications in the reflex organization of hindlimb muscle groups. We propose that the changes observed in GABA immunoreactivity of cells and terminals is an expression of changes in their modulatory activity to compensate for the alterations in the afferent information.

INTRODUCTION

The first section of this report will place emphasis upon some particular responses to weightlessness observed in the adductor longus muscle of rats flown in the Soviet COSMOS flight 2044, namely, 1) muscle fiber injury, 2) regenerative phenomena, and 3) alterations of the neuromuscular junctions. In previous studies, investigations carried out upon different muscles after both flight and ground-based (mostly hindlimb suspension) experiments have provided information on the effects of microgravity and "simulated" microgravity upon morphology, metabolic properties, histochemistry and electrophysiology (see Edgerton and Roy, for review, 1994). Through these studies we have learned that "slow" muscles, mostly composed of type I fibers (e.g., soleus, adductor longus), carry the burden of the changes while "fast" muscles, mostly composed of type II fibers (e.g., tibialis anterior) are relatively unaffected.

The second section of this report will deal with the possible consequences that limb unloading may have upon those areas of the central nervous system related to sensory inputs from muscles. Our assumption

—based on our current behavioral and morphological studies (D'Amelio *et al.*, 1987; D'Amelio and Daunton, 1992; Fox *et al.*, 1993, 1994)— was that muscle atrophy produced by limb unloading could modify sensory inputs arising from muscle receptors to the cerebral cortex. We focused our analysis on the behavior of GABAergic neurons of the hindlimb representation of the somatosensory cortex since numerous lines of research have demonstrated modifications in the level of GABA-IR or glutamic acid decarboxylase (GAD) immunoreactivity in cortical interneurons when sensory activity is altered by surgical manipulation (Hendry and Jones, 1986; Warren *et al.*, 1989; Akhtar and Land, 1991; see also Jones, 1990).

MATERIAL

Muscle Study

Wistar-derived male rats (SPF) from the Institute of Endocrinology, Bratislava, Czechoslovakia, aged approximately 3.5 months and weighing on average 180 grams at launch, were used in this experiment. Five animals per group (1 flight group and 3 control groups) were employed. The animals were not subjected to any type of invasive procedure. The flight animals remained for 14 days exposed to the space environment. Animal handling, launching details, as well as the procedures employed on muscle tissue have been described elsewhere (D'Amelio and Daunton, 1992).

Cerebral Cortex Study

Hindlimb unloading by tail suspension (HLS) to simulate some of the effects of weightlessness on muscles observed following spaceflight (SF) (see Ilyin and Oganov, 1989; Thomason and Booth, 1990; Edgerton and Roy, 1994, for reviews) was employed for this study. Six Sprague-Dawley rats (200-250 g) were employed. Three served as controls and three were suspended (HLS) by the tail for 14 days. The hindlimb representation of the somatosensory cortex was identified in Nissl-stained slides by the prominent aggregation of granular cells in layer IV. GABA-IR cell counts were done on pair of sections (control and experimental) on the same slides. Particulars of suspension procedure, perfusion of animals, immunostaining and methodology for quantitative analysis of GABAergic cells have been published elsewhere (D'Amelio *et al.*, 1996).

RESULTS

Muscle Study

The main alterations observed in all the flight animals, and not in any of the control animals, were myofiber atrophy, segmental necrosis (frequently accompanied by extensive cellular infiltration composed of macrophages, polymorphonuclear leukocytes and mononuclear cells) (Figure 1) and regenerating myofibers that were immunoreactive to N-CAM (Figure 2). For the quantitative assessment of myofiber atrophy Z band length was measured to approximate myofiber diameter in electron microphotographs. In the flight animals Z band length ranged from 1,460 Å to 2,600 Å with a mean of 2,095 Å while in the control animals the range was from 3,100 Å to 3,500 Å with a mean of 3,109 Å ($F(1,6) = 8.55$, $p = .0265$).

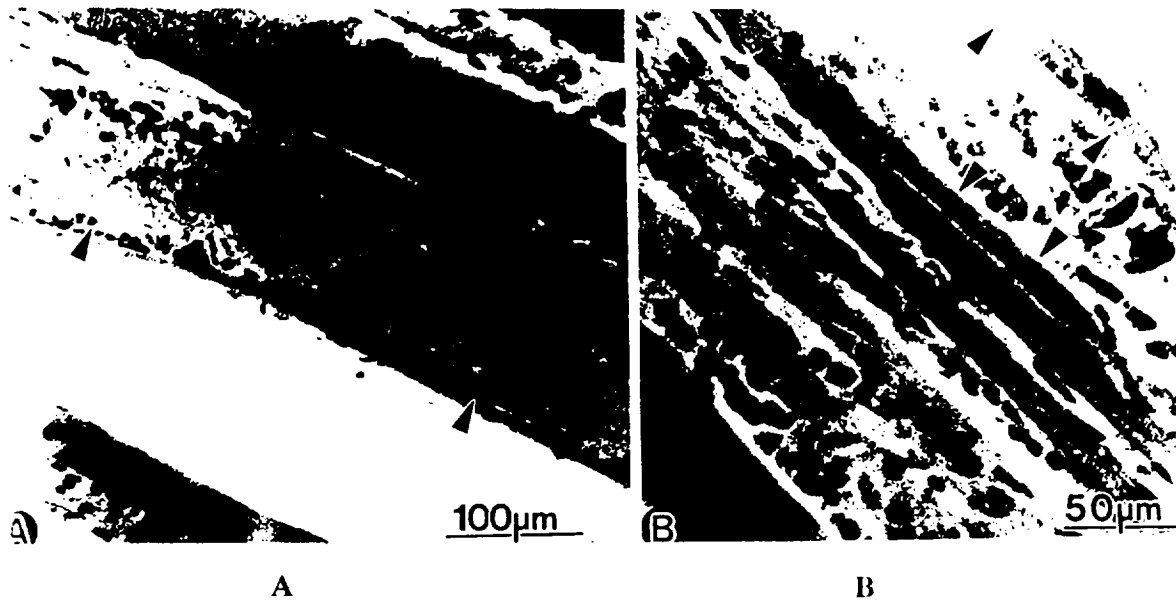


Fig 1. Flight animals. In (A), longitudinal sections show segmental necrosis of myofibers (arrowheads) accompanied by inflammatory cellular infiltration. In (B), atrophic fibers (arrowheads), edema and cellular infiltrates mainly composed of histiocytes and polymorfonuclear leukocytes. From D'Amelio and Danton (1992), with permission from the publisher.

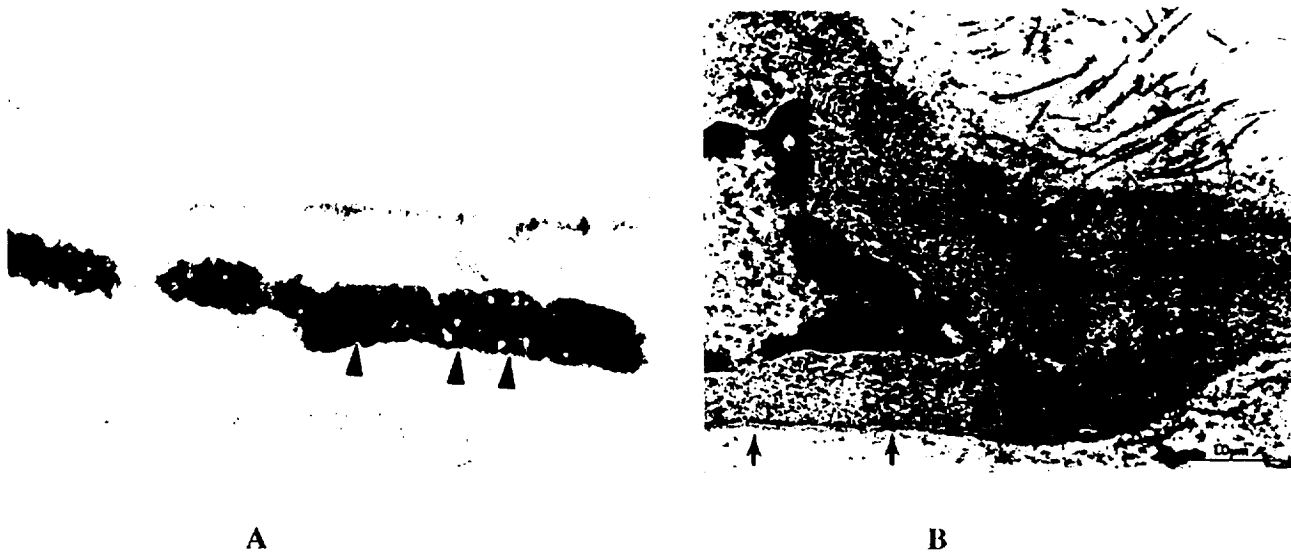
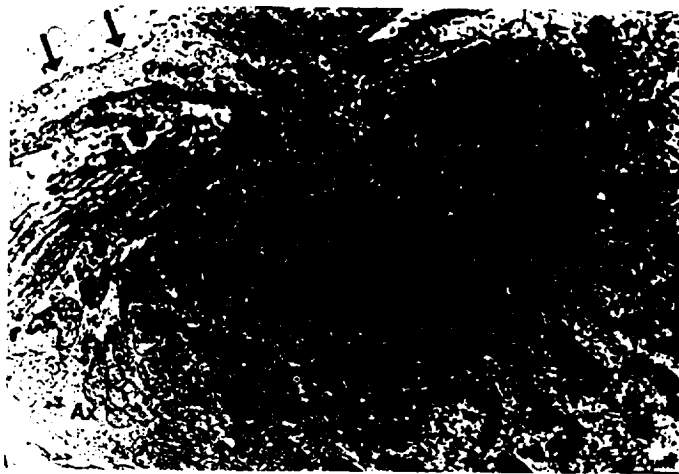


Fig 2. In (A) an N-CAM immunoreactive regenerating myofiber is shown. (B) High magnification of a regenerating myofiber reveals that the cytoplasm contains abundant ribosomal aggregates associated with bundles of still disorganized myofilaments (MF). Immature Z bands (Z) are also conspicuous. A visible basement membrane (arrows) surrounds the cell. From D'Amelio and Danton (1992), with permission from the publisher.

The most salient changes of the neuromuscular junctions were: absence of synaptic vesicles with replacement by microtubules and neurofilaments, interposition of Schwann cell processes between pre- and postsynaptic membranes, "unemployed" axonal spaces with shallow primary clefts, complete degeneration of axon terminals, and axonal sprouting (Figures 3 and 4). Of the 40 neuromuscular junctions from flight animals 24 (89%) showed one or more of these changes. In the 38 neuromuscular junctions from control animals only 11% showed one or more of these changes ($X^2 = 23.38$; $p < .0001$). No alterations of muscle receptors (i.e., muscle spindles) was seen in our preparations.



A

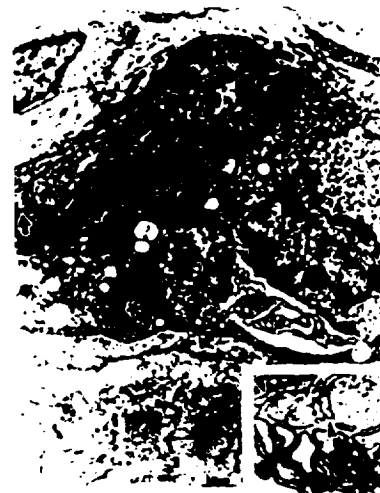


B

Fig. 3. (A) Synchronous control. Neuromuscular junction showing a preterminal axon (arrows) that gives rise to three axon terminals (Ax) apposing normal junctional folds. (B) Flight animal. The figure shows an axon profile almost devoid of synaptic vesicles and containing microtubular structures and few neurofilaments. From D'Amelio and Dauntton (1992), with permission from the publisher.



A



B

Fig. 4. (A) Flight animal. Neuromuscular junction displaying shrunken axon profiles (Ax1 and Ax2) occupied by myelin figures. Ax3 is completely devoid of synaptic vesicles. Schwann cell processes with degenerative alterations surround Ax1 and Ax2 (arrows) while Ax3 is covered by identifiable Schwann cell processes (arrowhead). (B) Flight animal. A myofiber undergoing necrosis (NF) shows dissolution of myofibrillar architecture, remains of altered myofibrils (*) and chromatin clumping and lysis of nuclei. A neuromuscular junction displays an elliptical axon profile (Ax) and junctional folds of apparently normal morphological characteristics. The reaction product of the synaptic cleft and junctional folds corresponds to esterase activity revealed by the staining procedure used to localize motor endplates. A small axon suggestive of an axonal sprout (arrow and inset) occupying the same post-synaptic space as the main axon terminal is separated from the latter by Schwann cell processes that also cover the sprout (arrowheads in inset). From D'Amelio and Dauntton (1992), with permission from the publisher.

Cerebral Cortex Study

The number of GABA-IR cells/mm² of the hindlimb representation was determined for each section lying within the boundary defined by the presence of the rostral hippocampus (Paxinos and Watson, 1986). A total of more than 7600 GABA-IR cells were identified. Cell counts on sections of HLS and control rats that were processed in the same immunostaining solutions were expressed for HLS as a percentage of control

$$\frac{(\text{HLS GABA-IR cells/mm}^2)}{(\text{CONTROL GABA-IR cells/mm}^2)} \times 100$$

GABA-IR cells were scattered in all cortical layers, but with the highest concentration in layer IV and lower concentrations in layers I and VI. The number of GABA-IR cells was reduced in rats subjected to HLS. Effects of HLS, expressed as the percentage of reduction in GABA-IR cells, in each cortical layer showed that the reduction in GABA-IR cells varied among cortical layers with significant reductions occurring in layers Va and Vb (32.75% and 22.07% respectively). Although quantitative assessment of GABAergic terminals ("puncta") targeting pyramidal cell soma and processes was not performed, it was obvious that they were markedly reduced in number in layers Va and Vb when compared with controls (Fig. 5).

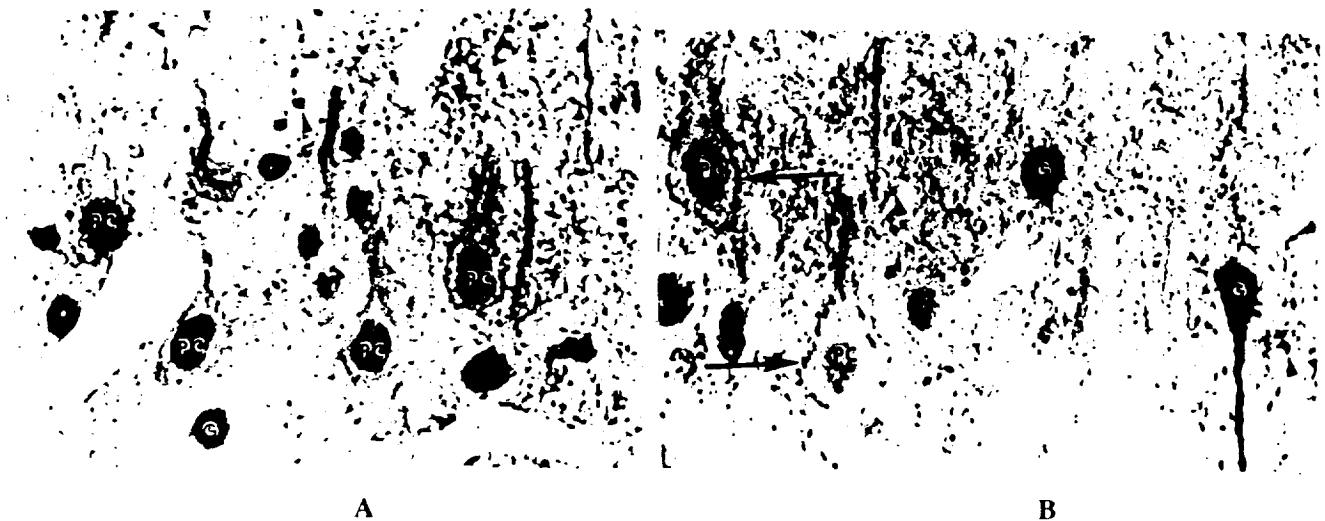


Fig. 5. Microphotographs of hindlimb somatosensory cortex at the level of layer Vb stained with GABA antiserum. (A) Tail-suspended animal. The pyramidal cells appear almost totally deprived of peripheral GABA-IR terminals. Note that the neuropil also shows very few terminals (arrowhead) as compared with the control in (B). (B) Control animal. Pyramidal cells surrounded by GABA-containing terminals (arrows). Numerous GABA-IR terminals are also conspicuous in the neuropil (arrowheads). PC, pyramidal cell; G, GABAergic cell. Magnification: 800x. From D'Amelio *et al.* (1996), with permission from the publisher.

DISCUSSION

A prolific literature exists on the numerous factors involved in triggering the process of muscle atrophy and subsequent deterioration of the myofibrillar structure in conditions of microgravity. The structural and metabolic foundations underlying these changes have been reviewed by Ilyin and Oganov (1989). Denervation-induced changes at the neuromuscular junctions have been reported in both spaceflight and ground based experiments (hindlimb unloading by tail suspension) as well (Riley *et al.*, 1990; Il'ina-Kakueva and Portugalov, 1977; Baranski *et al.*, 1979; D'Amelio *et al.*, 1987; Pozdnyakov *et al.*, 1988). It is interesting to note that most of the alterations that we have described in the adductor longus muscle must have taken place

during spaceflight and not as a consequence of post-flight exercise since the flight animals were sacrificed within approximately 3-11 hours after landing. It has been shown that it takes 2-3 days for typical

at the cortical level. In these modifications local circuit GABAergic neurons of the cerebral cortex are the most logical candidates to modulate the discharge frequency of pyramidal cells (see Jones, 1993) since: a) The majority of identified local circuit neurons in the cerebral cortex are GABAergic (White, 1989), b) GABAergic cells are present in all layers of the mammalian cerebral cortex (Ribak, 1978; Houser *et al.*, 1984; White, 1989) and c) The main synaptic target of all classes of GABAergic neurons are the pyramidal cells and their processes (White, 1989). Furthermore, experiments primarily concerned with neuronal receptive fields in the somatosensory cortex have shown that GABA-mediated intracortical inhibition specifies size and thresholds of receptive fields of major neuronal subgroups (Hicks and Dykes, 1983; Dykes *et al.*, 1984; see also Jacobs and Donoghue, 1991). It has been shown that the cortical substrate subserving tactile and proprioceptive limb placing—that is deeply disturbed after HLS (Fox, unpublished data)—coincide with a dense subfield of large pyramidal neurons in the deeper part of layer V (De Ryck *et al.*, 1992). In our experiments, layer V showed the most pronounced reduction of GABA-IR cells.

In short, as a result of the selective and differential effects of HLS on weight and non-weight bearing muscles, corticospinal fibers would influence motoneuronal pools with either a significant number of abnormal axon terminals innervating the atrophic antigravity muscles or with normal axon terminals innervating non-weight bearing muscles having minimal or no alterations. As a consequence, disturbances in the afferent signaling and feedback information from intramuscular receptors (particularly muscle spindles) to the cerebral cortex would trigger an imbalance in the reflex organization of these synergetic muscle groups. In turn, pyramidal tract neurons processing altered sensory information would respond with changes in the rates of discharge that are modulated by GABAergic neurons.

The emphasis put on muscle spindles over other receptor types as responsible for the changes has a reason, although admittedly speculative. Electrophysiological studies of the rat somatosensory cortex suggest an overlap (co-extension) of sensory and motor areas ("sensorimotor amalgam"), *particularly at the level of the hindlimb representation* where layer V contains large pyramidal cells that extend over, without interruption, from the motor cortex (Hall and Lindholm, 1974). This type of cortical organization would seem to lend support to the hypothesis first proposed by Phillips (1969) that information from muscle spindles to the cerebral cortex is relayed through an oligosynaptic transcortical spindle circuit for proprioceptive signals whose efferent limb is the corticomotoneuronal projection (see Hummelsheim and Wiesendanger, 1985, for discussion). Several subsequent studies have provided more evidence in favor of this hypothesis (see Landgren and Silfvenius, 1969, 1971; McIntyre, 1974; Wiesendanger and Miles, 1982; Matthews, 1991). Whether the decrease in GABA immunoreactivity is due to alterations in its synthetic activity or depletion due to increased release is a matter of speculation that will require additional studies (e.g., *in situ* hybridization). Furthermore, electrophysiological recordings will be necessary to assess patterns of activity and receptive field size of cortical neurons influenced by GABA-mediated inhibition under the same conditions. Since the changes we have described are presumably transient (normal gait is recovered after several weeks-Fox *et al.*, 1993, 1994) it would be important to investigate changes in GABA-IR during the recovery process, and to assess whether these alterations may become irreversible given a sufficiently long period of hindlimb unloading.

Changes in GABA-IR were previously reported under conditions of sensory deprivation by surgical means. For example, Warren *et al.* (1989) reported a 16% decrease of glutamic acid decarboxylase (GAD) immunoreactive cells in layer IV of the rat hindlimb somatosensory cortex 2 weeks after transection of the sciatic nerve. In experiments conducted in monkey visual cortex after 2-3 weeks of eye enucleation, Hendry and Jones (1986) found a 45% reduction of GABA-IR cells in layer IV. In the same region, these investigators also showed a 36% decrease of GABA-IR cells 11 weeks after eyelid suture.

Unlike surgical deafferentation, in HLS the afferent input is not interrupted but rather significantly disrupted by non-invasive unloading of weight-bearing muscles. Our results suggest that non-invasive manipulations of the neuromuscular system, e.g., HLS or SF, can have significant effects on cortical circuitry. Other lines of work based on non-invasive procedures support this possibility (see for example, Jenkins *et al.*, 1990; Merzenich *et al.*, 1990; Sanes *et al.*, 1992). Since the central nervous system must constantly adjust movements in response to altered environmental conditions, we believe that studies in intact animals should be pursued to help clarify the mechanisms of cortical plasticity and adaptation under natural conditions.

mononucleated myoblasts to appear after muscle injury (Snow, 1977; Nichols and Shafiq, 1979). We believe that the extensive necrosis, with the possible overlapping effects of the denervation-reinnervation process, are the triggering factors for myofiber regeneration. In addition, the presence of innervation on regenerating myofibers suggests a process of remodeling of axon terminals. Axonal regeneration expressed by the visualization of small axon terminals (sprouting) was also seen on some necrotic fibers.

The presence of microtubules and neurofilaments found in some axon terminals almost totally devoid of synaptic vesicles is also intriguing. It seems reasonable to speculate that such appearance might be another indication of axonal remodeling. Such remodeling may be related to variations in the metabolism of motoneurons that trigger a reversal from a "transmitting" (stable) to a "growing" (plastic) state (Watson, 1976; Gordon, 1983). It has been shown that microtubules predominate during development and that during the regenerative response of motoneurons there is an increase in the ratio of tubulin to neurofilament which expresses a recapitulation of the more plastic states that take place during development (Hoffman and Lasek, 1980; Lasek, 1981).

The alterations of the neuromuscular junctions described in this report seem to suggest a process of denervation and remodeling during spaceflight, that is to say, a process limited to the "efferent" component of muscle innervation. Pronounced myofiber atrophy of antigravity muscles accompanied by severe alterations in a significant number of motor units have also been previously reported in HLS (D'Amelio *et al.*, 1987; see Edgerton and Roy for review, 1994). These findings, however, only represent a fragmentary view of the response of the neuromuscular system to spaceflight or HLS. Thus, we believed that further research in this area would profit from the development of a more "systemic" approach that would address questions such as, for example, what are the "functional" and/or morphological alterations of the "afferent" component of muscle due to the extensive lesions of the myofibers, and what are the effects on areas of the cerebral cortex related to inputs from muscle receptors. A natural result of this "systemic" approach would be a more thorough understanding of the adaptive capabilities of the organism to altered gravitational conditions. We thought then appropriate, as a following step, to initiate correlative studies on the most plastic structure of the central nervous system, the cerebral cortex, in animals subjected to "simulated" microgravity (HLS).

Consequences of limb unloading at the level of the cerebral cortex after spaceflight or HLS have not previously been addressed. Several lines of evidence lead us to suggest that the cortical changes reported here—reduction of GABA-IR cells and terminals in layer Va and Vb of the rat hindlimb somatosensory cortex—result from altered proprioceptive inputs from hindlimb muscle receptors with the possible participation of joint receptors and tendon organs. First, despite the changes described by us and others in muscle fibers and neuromuscular junctions, no morphological changes in muscle spindles or other sensory structures have been revealed by either light or electron microscopic observations. It is therefore likely that after HLS or SF sensory receptors continue to convey signals to the cerebral cortex from "slow" weight bearing muscles (e.g., soleus, adductor longus), as well as from the predominantly "fast" non-weight bearing muscles (e.g., tibialis anterior) of the hind limbs.

Second, since receptors of the affected "slow" extensors (e.g., soleus) and the relatively unaffected "fast" extensors (e.g., lateral and medial gastrocnemius) and "fast" flexors (e.g., tibialis anterior) apparently remain operative following HLS or SF, a mismatch of afferent messages from these muscles to the cerebral cortex should be expected. Since in normal conditions stretching of the antigravity soleus muscle evokes heterogenic reflexes in lateral and medial gastrocnemius and tibialis anterior (Nichols, 1989; see also Cope *et al.*, 1994), an imbalance in the reflex responses of these synergetic muscles is most likely responsible for the disruption of gait previously demonstrated by us following HLS and SF (Fox *et al.*, 1993, 1994). That such an imbalance can lead to changes in the cerebral cortex has been demonstrated by Sanes *et al.* (1992). These investigators have suggested that sensory inputs from muscle receptors are used to adjust the neural circuits related to the specific output functions of the motor cortex and that a mismatch between cortical outputs and sensory inputs during active limb movements (e.g., during walking) can lead to the reorganization of the cortical motor outputs. Such goal-directed reorganization would be designed to optimize function (e.g., walking) under the conditions of altered inputs from hindlimb muscles.

Thus, the modification of sensory inputs to the central nervous system due to altered functioning of hindlimb muscles, along with the requirements for reprogramming of motor outputs to compensate for the changes in structure and function of those same muscles, could lead to plastic modifications of the circuitry

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Light microscopic image analysis system to quantify immunoreactive terminal area apposed to nerve cells

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Abstract

The present report describes a desktop computer-based method for the quantitative assessment of the area occupied by immunoreactive terminals in close apposition to nerve cells in relation to the perimeter of the cell soma. This method is based on Fast Fourier Transform (FFT) routines incorporated in NIH-Image public domain software. Pyramidal cells of layer V of the somatosensory cortex outlined by GABA immunolabeled terminals were chosen for our analysis. A Leitz Diaplan light microscope was employed for the visualization of the sections. A Sierra Scientific Model 4030 CCD camera was used to capture the images into a Macintosh Centris 650 computer. After preprocessing, filtering was performed on the power spectrum in the frequency domain produced by the FFT operation. An inverse FFT with filter procedure was employed to restore the images to the spatial domain. Pasting of the original image to the transformed one using a Boolean logic operation called 'AND'ing produced an image with the terminals enhanced. This procedure allowed the creation of a binary image using a well-defined threshold of 128. Thus, the terminal area appears in black against a white background. This methodology provides an objective means of measurement of area by counting the total number of pixels occupied by immunoreactive terminals in light microscopic sections in which the difficulties of labeling intensity, size, shape and numerical density of terminals are avoided. © 1997 Elsevier Science B.V.

Keywords: Image analysis; FFT; NIH-image; Quantitative immunocytochemistry; GABA; Somatosensory cortex; Light microscopy

1. Introduction

The quantitative assessment of antibody immunocytochemistry in light microscopic sections presents well known difficulties. These include non linearity of optical density measurements of immunoreactive products, uneven lighting and subjective evaluation of staining intensity. In the course of our research (D'Amelio et al., 1996) we explored the possibility of decreasing subjective bias by using a computer-based image analysis technique to measure the area (in pixels) occupied by

immunoreactive terminals in close apposition to nerve cells. Other approaches with similar purposes have previously been reported (Vincent et al., 1994).

2. Material and methods

2.1. Animals, perfusion fixation and sectioning

Sprague-Dawley rats (200–250 g) were employed for this study. The animals were deeply anesthetized with Metophane[®] and immediately perfused via the heart with 50 ml of 0.9% saline, followed by 500 ml of a fixative made up of 1% paraformaldehyde and 2%

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glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed the same day, immersed in fresh fixative and stored at 4°C. The right hemisphere was coronally blocked between Bregma –1.8mm and Bregma –3.6mm, where the somatosensory representation of the hindlimb is conspicuous and associated with the presence of the rostral hippocampus (Paxinos and Watson, 1986). Coronal sections 40µm thick were cut on a Vibratome® and collected in TBS (0.05 M Tris buffer, 0.9% saline, pH 7.6).

2.2. Immunocytochemistry

The tissue sections, both experimental and control, were processed together in the same solutions to minimize labeling differences.

Floating sections were incubated for 5–10 min at room temperature (RT) with 3% hydrogen peroxide in 10% methanol in TBS and subsequently rinsed four times in TBS × 30 min (RT). The sections were then immersed in GABA antiserum (Chemicon, Cat. # AB131) or control serum (preimmune rabbit serum) diluted at 1:1000 in TBS for 48–72 h at 4°C, with orbital agitation. Then, they were rinsed four times in TBS × 30 min (RT) and incubated for 60 min (RT) in swine anti-rabbit IgG diluted 1:50 in TBS. The sections were rinsed four more times in TBS × 30 min (RT) and then incubated for 60 min (RT) with rabbit peroxidase–antiperoxidase complex (Sigma) diluted 1:200 in TBS. To develop reaction product the sections were immersed in 12.5 mg diaminobenzidine tetrahydrochloride (DAB) in 50 ml TBS + 5 µl 30% hydrogen peroxide for 5–8 min. Finally, the sections were rinsed in TBS, two changes × 10 min (RT), mounted on gelatin coated slides, air-dried and coverslipped with Permount®.

2.3. Image analysis equipment

2.3.1. Light microscope

Sections were observed under a light microscope (Leitz Diaplan) equipped with a 100 W halogen lamp and with a Fluotar 100/1.32 oil immersion objective. Two filters (a Kodak Polycontrast photographic filter #11/2 and a Wratten gelatin filter #15, deep yellow) were placed in the microscope light path to enhance contrast and increase accuracy of focus.

2.3.2. Image analysis system

Images were captured using a Sierra Scientific (Sunnyvale, CA) Model 4030 CCD camera. This is a black and white video-rate camera with 640 horizontal scan lines and 492 vertical scan lines. It was mounted on the microscope body connected to a Scion Technology (Friederick, MD) LG-3 frame grabber board installed in a Nubus slot in a Macintosh Centris 650

computer (Cupertino, CA). The LG-3 board samples the analog video signals from the camera into a 640 × 480 grid of pixels with a resolution of eight bits. The brightness level of each pixel ranges from 0 to 256 gray levels as it is converted into the digital image. The public domain software, NIH-Image v. 1.59 (written by Wayne Rasband, NIMH, Bethesda, and updated frequently), was used to capture images and to analyze the GABA-IR terminals. This software is available electronically from the Internet by anonymous FTP from zippy.nih.nih.gov/pub/nih-image/nih-image or from the NIH's Web site (<http://rsb.info.nih.gov/nih-image>).

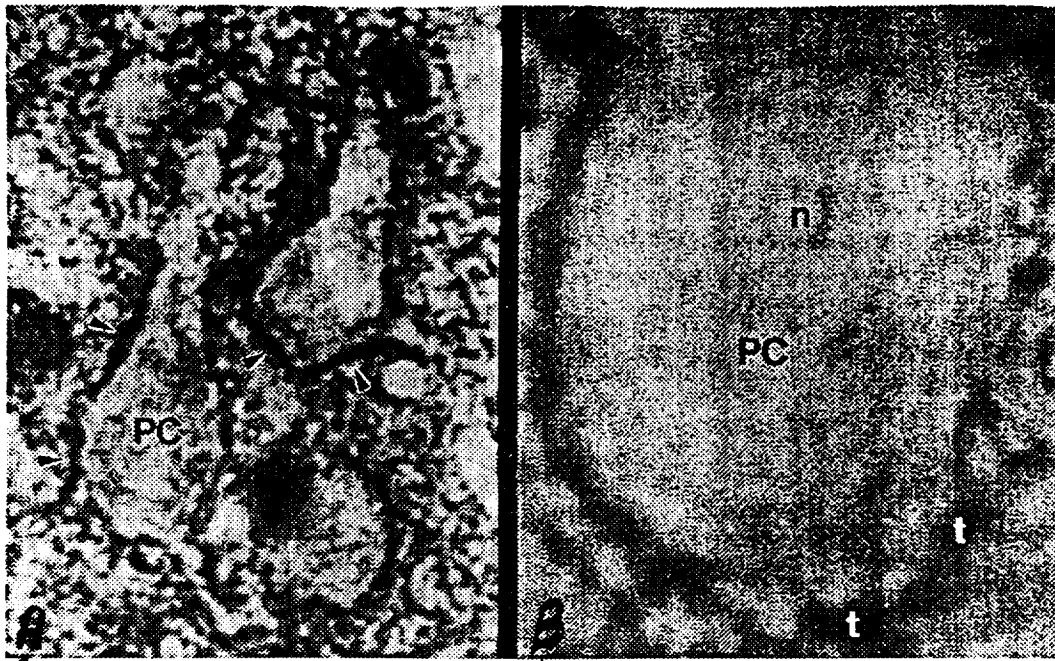
2.4. Image processing steps

2.4.1. Image capture

Our analysis was focused on GABA-immunoreactive (GABA-IR) terminals closely apposed to pyramidal cells of layer V of the somatosensory cortex. Pyramidal neurons were identified by round or oval contours and a distinct apical dendrite. No GABA-IR product was present in the soma of these cells.

Once a pyramidal cell was selected to be analyzed, the light source intensity for the microscope and the video control menu (gain and offset) under NIH-Image were adjusted until the peak intensity of the gray level displayed from the live histogram was close to the midpoint of the range between 0 and 255. Then, the microscope stage was moved off the tissue without changing any video control settings, and a blank field was captured. The latter was stored in the temporary memory of the system. Subsequent cell images, under software control, were captured 16 times and then were averaged to reduce random electronic noise originated from various sources including the camera's CCD sensors, frame grabber, and monitor (Inoué, 1986). The software automatically subtracted the blank field from the averaged images before the final images were captured by the frame grabber to further improve the signal to noise ratio. Thus, GABA-IR terminals in the captured images appeared to stand out better and random noise was reduced. To maximize use of computer storage space, the final captured images were cropped to the size of each pyramidal cell, usually at least 260 × 300 (horizontal × vertical) pixels in size, and saved. Further image processing and analysis was performed on those cropped images. Neurons from both control and experimental sections were captured without changing light and video settings.

A light microscopic microphotograph under oil immersion depicts pyramidal cells outlined by GABA-IR terminals (Fig. 1A). Fig. 1B shows the captured and cropped digital image of one cell.



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Fig. 1. (A) Microphotograph of pyramidal cells (PC) outlined by GABA-IR terminals (arrowheads) in layer V of the hindlimb representation of the rat somatosensory cortex. Magnification is $600\times$. (B) Cropped digital square image of a pyramidal cell soma (PC) outlined by GABA-IR terminals (t) captured from a microscopic slide viewed under $100\times$ oil immersion objective; n, nucleolus.

2.4.2. Preprocessing of the digital image

Under the Process menu in the software, a type of neighborhood ranking operation—median filter with a 3×3 pixel matrix—was used to reduce electronic noise in the captured image. This filter sorts the nine pixels in each 3×3 neighboring region and replaces each center pixel from the source image by the median value of its eight neighbors. The effect is to remove all pixels that are darker or brighter than their neighbors, and thus remove noise. This is a linear filter operation in which no information is lost from the original image (Russ, 1994). Following median filtering, a sharpening process (also under the Process menu) to enhance the boundaries of terminals was applied.

Fig. 2 shows a flow chart of the image processing steps.

2.5. Image analysis steps

2.5.1. Fast Fourier Transform (FFT)

Fourier Transform (FFT) routines were employed to analyze immunoreactive terminals outlining pyramidal cells in layer V of the hindlimb representation of the somatosensory cortex. The algorithm used in NIH-Image v.1.59 and subsequent versions uses the computationally advantageous Fast Hartley Transform or FHT, (Bracewell, 1986), a close relative of the well known Fast Fourier Transform. The FHT was originally implemented by Arlo Reeves (1990) in his spin-off version of Image FFT. These routines were written in assembly language specific for the 68000 processor for v.1.28 of

NIH-Image. They have now been adapted to current chip technology in v.1.59.

2.5.2. FFT macros

FFT macros are invoked initially using 'Load macros' under the Special menu. A square area, comprised of 128×128 or 256×256 pixels (the size of this selected area must be a power of two, a requirement of the FFT), was selected by applying one of the procedures in the FFT macros. The FFT was performed on a square area of the image to obtain a power spectrum image in the frequency domain (Fig. 3). Different spatial signals from the original image were represented as different frequencies at various distances from the center of the power spectrum, with concentration of lowest frequencies closer to the center, and the higher frequencies further away from the center.

2.5.3. Inverse FFT

A software filter (under FFT macros in the Special menu), size 80% (retaining 80% of the original frequencies), and a transition zone, with a width of 20%, was created and applied to all cells for analysis (screen display in Fig. 4). When an inverse FFT with filter procedure (under FFT macros) was employed, the information in the frequency domain is transformed back to the spatial domain (screen display in Figs. 4 and 5). This operation restores the original image with the high frequencies suppressed, making the image of the terminal area more prominent.

2.5.4. Boolean 'AND'ing and thresholding

In this step the original image was pasted to the transformed image using the Boolean logic operation 'AND' (under 'Paste control' option in Windows menu), so that the terminals in focus were clearly delineated from the background (screen display in Figs. 6 and 7). If a cell was larger than the 256×256 area, a composite of squares was made, using Boolean logic 'OR' under 'Paste control' to match the squares. As to the threshold, instead of having to adjust it according to each individual image, in this application the threshold end point was set at 128 for all images. This end point consistently selected all pixels of the terminals, whereas in settings beyond 128 many pixels would remain unselected. A binary image was then created that revealed the terminal area in black against a white

Image Processing Steps

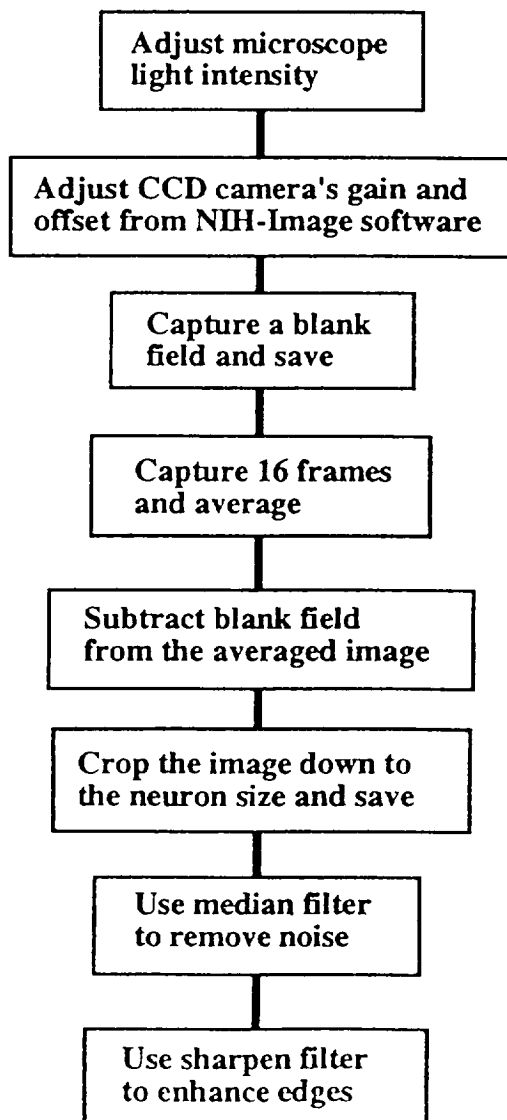


Fig. 2. Flow chart of the image processing steps.

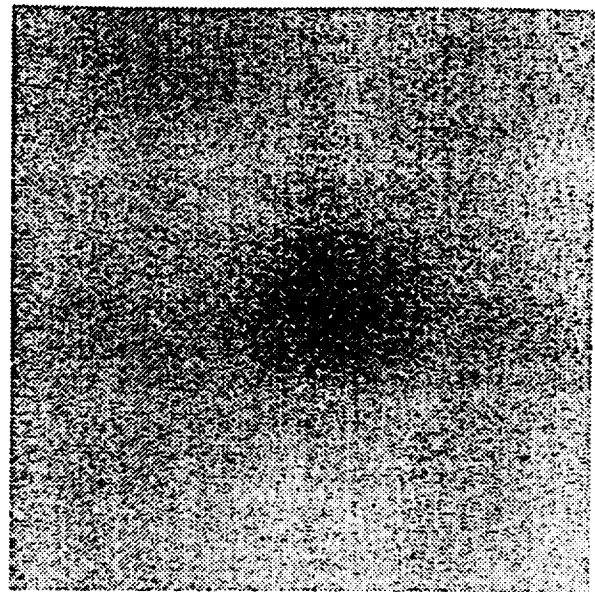


Fig. 3. Power spectrum of a pyramidal cell produced by FFT of a square image in the frequency domain.

background (Fig. 8).

2.5.5. Measurements

The terminal area was measured on binary images and the perimeter of the cell bodies was estimated on gray scale images. The PENCIL tool in the software was employed to separate the clearly delineated terminals in close apposition to the pyramidal cell from those axon terminals that were not apposed (Fig. 8). To measure the area of the terminal, the WAND tool, an automatic measuring tool (highlighted in Tools window in Fig. 8), was employed to count the number of pixels in the black zone, when the area measurement option in the software was selected. With the shift key depressed, individual measurements were added together. The perimeter of the pyramidal cell body was estimated by using the POLYGON tool to trace the outline of the soma (Fig. 9). The dendritic gaps (apical and basal) were subtracted from the perimeter measurement.

A flow chart of the image analysis steps is shown in Fig. 10.

2.6. Statistical analysis

All measurements were exported directly into Excel (Microsoft, Redmond, WA) for easy record keeping, and for easy computation of the ratio of the area of the terminal to the perimeter of the soma. The ratios from all the cells analyzed were then exported into SuperANOVA (Abacus, Berkeley, CA) and a one-factor ANOVA was used to evaluate the effect of different experimental conditions on the area occupied by GABA-IR terminals apposed to pyramidal cells.

Super-
ANOVA
ANOVA

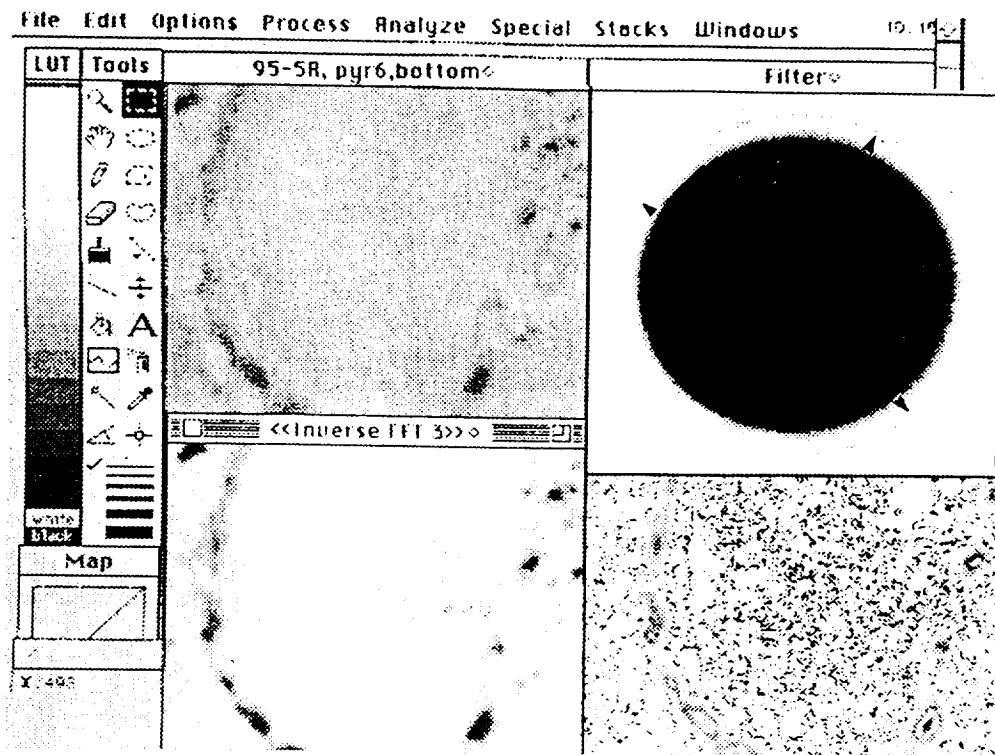


Fig. 4. Screen display of NIH-image software. Upper left corner: square image of a pyramidal cell; upper right corner: filter [size: 80% in black; 20% transition width (arrowheads)]; lower left corner: pyramidal cell soma outlined by GABA-IR terminals after inverse FFT with filter; lower right corner: resulting image from Boolean 'AND' ing the two images from the left side of the screen.

3. Example of data analysis

As part of an ongoing project in our laboratory, the procedure that has been described was employed to analyze the area occupied by GABA-immunoreactive terminals apposed to pyramidal cells in layer V of the

hindlimb representation of the rat somatosensory cortex following 14-day exposures to chronic hypergravity (3 G) produced by centrifugation. A significant reduction in the GABA-immunolabeled terminal area was found with respect to the control group. A total of 100 pyramidal cells, each from the control group and from the rats exposed to hypergravity were analyzed. The ratio of the area of GABA-IR terminals to perimeter of pyramidal cell soma was 8.122 ± 0.259 (mean \pm S.E.M.) for the control and 5.008 ± 0.206 for the hypergravity group ($P < 0.0001$). These results demonstrate that the method is effective in determining quantitative differences in immunoreactive terminals (Fig. 11).

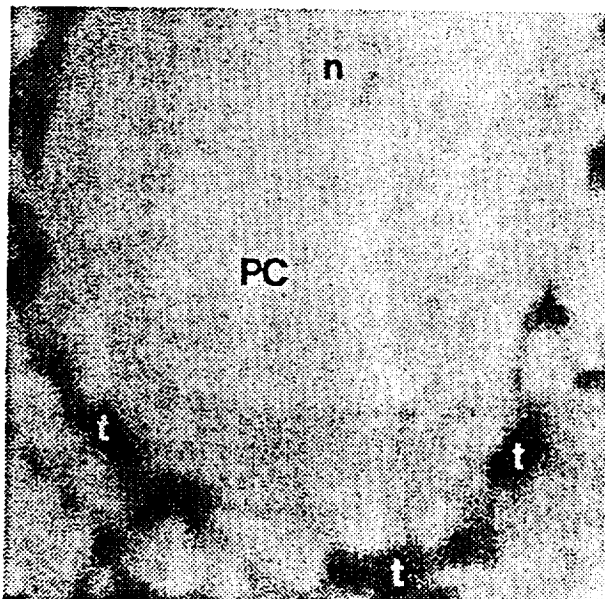


Fig. 5. Resulting image from applying inverse FFT with filter (size: 80%, transition width 20%) restoring the power spectrum image to the spatial domain. PC, pyramidal cell; t, terminals; n, nucleolus.

4. Discussion

The FFT applied to two-dimensional images is useful for various purposes, such as removing noise for image restoration, finding the periodicity in biological specimens, or for image enhancement to remove motion blur. For example, Russ (1994) has pointed out that a typical image analyst often avoids analysis in the frequency domain because of difficulties in relating the questions to be asked to the problems encountered in image analysis procedures. But with modern day software such as NIH-Image 'one does not need to deal deeply with the mathematics to arrive at a practical

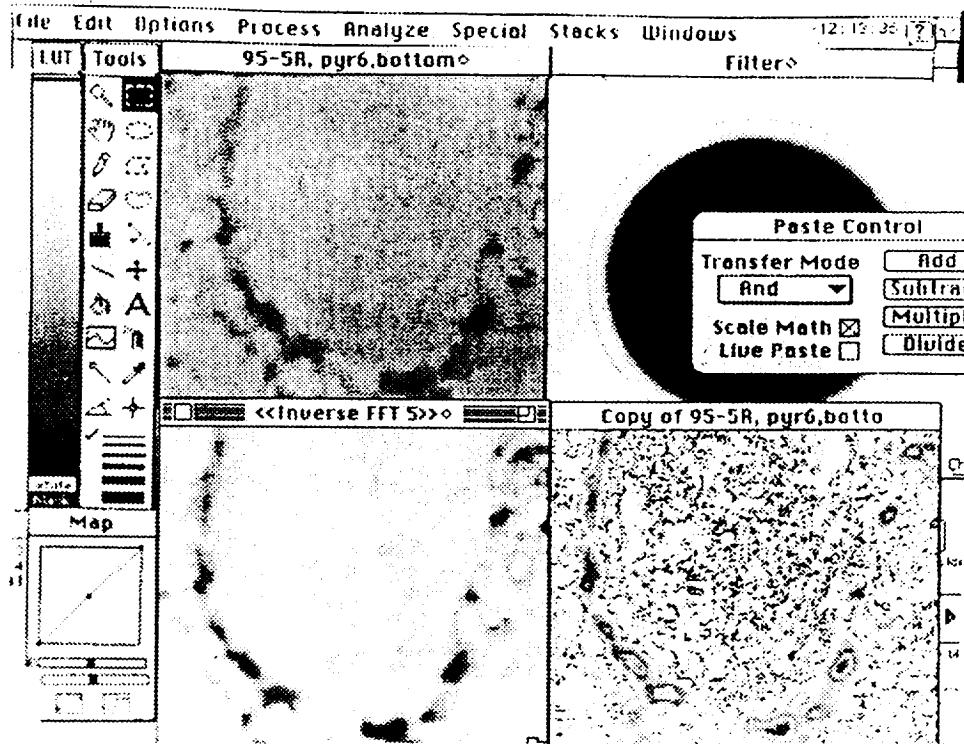


Fig. 6. Screen display showing paste control window with Boolean 'AND' selected. The rest of the screen is similar to Fig. 4.

working knowledge of these techniques' (Russ, 1994). An important property of FFT is that it can be reversed. The inverse FFT applied to the 'forward' FFT,

i.e., the power spectrum of an image, restores the original image. Filtering in the frequency domain before applying the inverse FFT, such as we did with the creation of the filter in the image analysis steps, removes most of the noise in higher frequencies. The

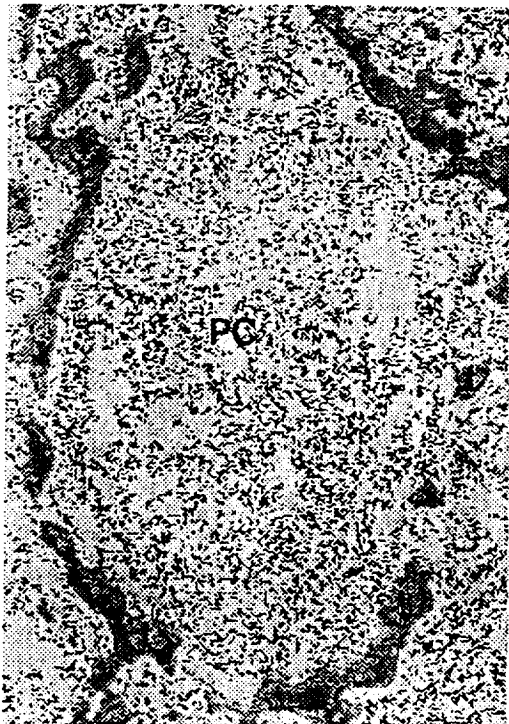


Fig. 7. The resulting image from Boolean 'AND'ing the square image (Fig. 1B) to the inverse FFT image (Fig. 5). GABA-IR terminals are clearly seen in the foreground. PC, pyramidal cell soma; t, terminals

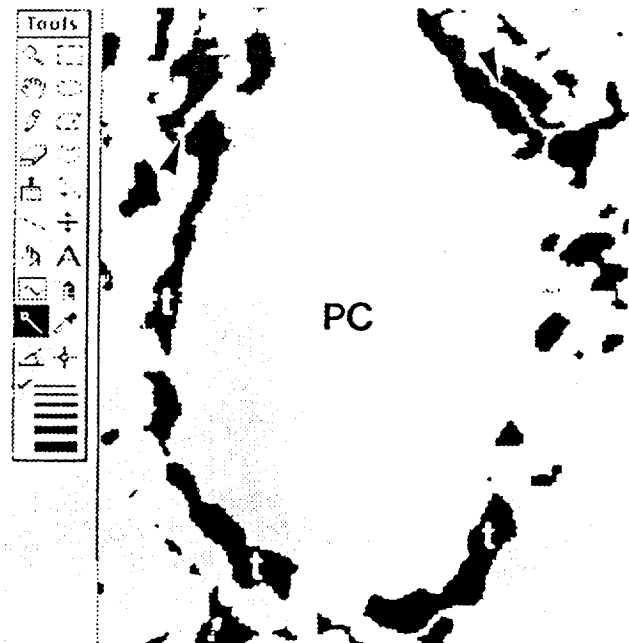


Fig. 8. Binary image after thresholding at 128 showing terminal area in black apposed to a pyramidal cell in white. Axon terminals not apposed have already been separated (arrowheads). The WAND tool is highlighted in Tools window. PC, pyramidal cell soma; t, terminals

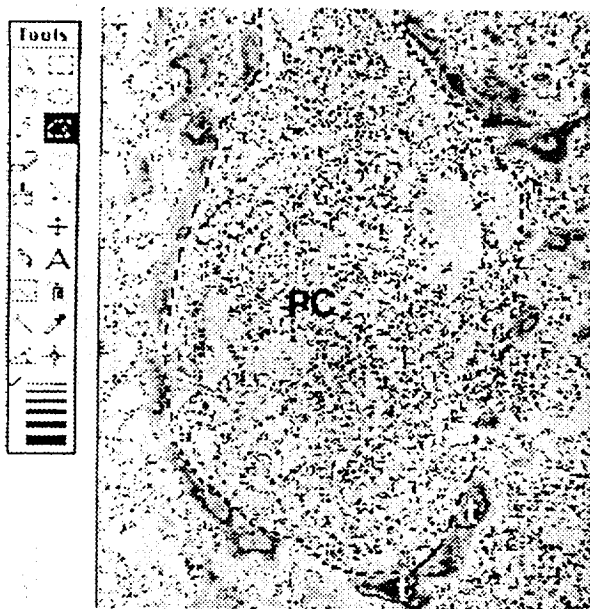


Fig. 9. Gray scale image of pyramidal cell soma (PC) with perimeter outlined employing the POLYGON tool (highlighted in Tools window); t, terminals.

analysis of images in the frequency domain (by application of FFT), was more efficient than processing in the spatial domain. The latter would have required many different steps of filtering and image mathematics with less satisfactory results. Using the inverse FFT with filtering, and then using the Boolean logic operation, 'AND'ing from the original image to the transformed image is essentially adding the signals together, thus bringing out the terminals into the focal plane and clearly differentiating them from the background. Furthermore, there is a definite endpoint of threshold at 128 that avoids subjective manipulation of threshold that could affect the results. Thresholding is a common step applied to the digital image before measuring. The traditional approach is to define a range of brightness values in the original image so that all the pixels within this range are selected as belonging to the foreground and measured, while all the other pixels belonging to the background are rejected. Since for practical reasons a large number of cells are analyzed over a period of several days, image capture from individual sections is often performed on different days. Thus, changes in lighting conditions may occur. However, after the FFT procedure is applied, a uniform threshold set at 128 can be employed for each cell from any section regardless of variations in lighting conditions.

We believe that the procedure described in the present report is useful since it increases the accuracy of the analysis by decreasing subjective interpretation and avoiding the difficulties and shortcomings presented by, for example, the quantitative evaluation of optical density in samples stained with immunocytochemical methods. These include variations in labeling intensity, the

need for a strict control of antibody concentration and times of incubation, the possibility of tissue alterations such as the compression of labeled profiles into smaller areas that may result in erroneous determinations of density and the use of standards containing a known

Image Analysis Steps

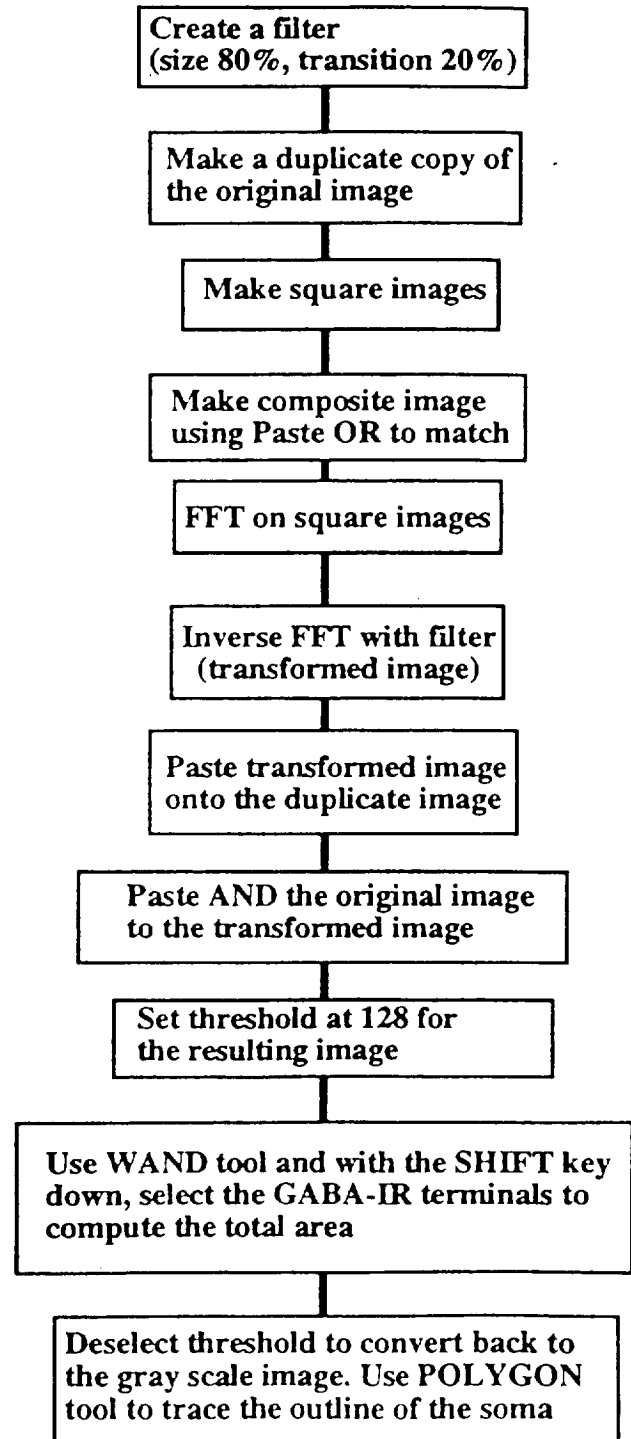


Fig. 10. Flow chart of the image analysis steps

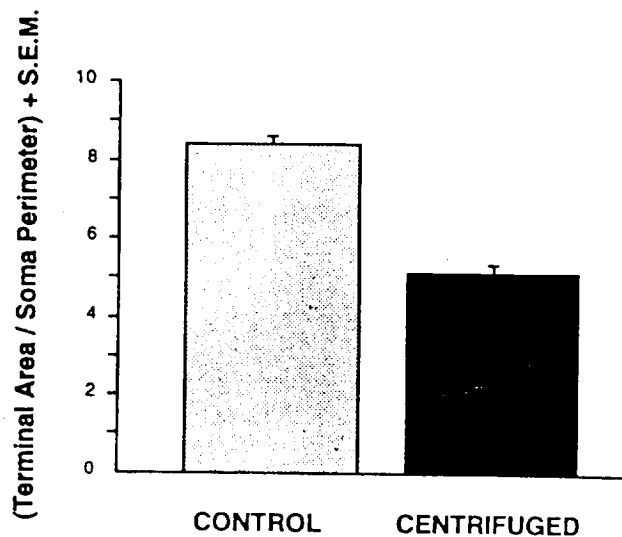


Fig. 11. GABA-immunoreactivity expressed as the ratio of terminal area to perimeter of the pyramidal cell body for animals in the control and centrifuged groups.

amount of antigen, since differences in optical density may not reflect changes in the concentration of the antigen (Mize, 1989, 1994). These drawbacks are further confounded by the possibility of uneven lighting during image capturing and monitor display that, with our procedure, are less important variables. The same can be said of labeling intensity of immunoreactive products. Although all steps of the immunocytochemical staining for both control and experimental sections were performed in the same solutions to avoid variations in labeling intensity, our procedure allows for such variations to occur without significantly impacting the results. As many researchers have learned, optical density measurements on immunoperoxidase products by means of light microscopy requires a sophisticated image analysis system. To perform those measurements, gray scale images have to be converted into binary images through thresholding. Thresholding that would be optimal for one area, would invariably be unsatisfactory for other areas. Thus, some of the needed features might be rejected or many of the background pixels might be included leading to erroneous result.

In the final analysis, the methodology to be employed should depend upon the questions to be answered. In our research we are interested in determining differences in the area occupied by GABA immunolabeled terminals apposed to pyramidal cells in regions of the somatosensory cortex (e.g. hindlimb representation) re-

lated to proprioceptive inputs from muscles. In this case we believe that, for example, the concentration of the antigen, important for the quantitative assessment of optical density, is less significant. More relevant to our purposes, if differences in GABA immunoreactivity are found between control and experimental samples, is to search for alterations in the synthetic activity of the transmitter.

Finally, we wish to emphasize some of the advantages of our procedure. They include the use of a common desktop computer, relatively inexpensive equipment, readily available free software to attain quantitative analysis, a standard procedure that can be easily followed, and minimal training requirements. (Expert help is also available from the NIH-Image, e-mail group located in soils.umm.edu). The methods described in this paper should well serve the purposes of others attempting to answer scientific questions of a similar nature.

Acknowledgements

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Quantitative Changes of GABA-Immunoreactive Cells in the Hindlimb Representation of the Rat Somatosensory Cortex After 14-Day Hindlimb Unloading by Tail Suspension

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The present study was aimed at evaluating quantitatively γ -aminobutyric acid (GABA) immunoreactivity in the hindlimb representation of the rat somatosensory cortex after 14 days of hindlimb unloading by tail suspension. A reduction in the number of GABA-immunoreactive cells with respect to the control animals was observed in layer Va and Vb. GABA-containing terminals were also reduced in the same layers, particularly those terminals surrounding the soma and apical dendrites of pyramidal cells in layer Vb. On the basis of previous morphological and behavioral studies of the neuromuscular system of hindlimb-suspended animals, it is suggested that the unloading due to hindlimb suspension alters afferent signaling and feedback information from intramuscular receptors to the cerebral cortex due to modifications in the reflex organization of hindlimb muscle groups. We propose that the reduction in immunoreactivity of local circuit GABAergic neurons and terminals is an expression of changes in their modulatory activity to compensate for the alterations in the afferent information. © 1996 Wiley-Liss, Inc.

Key words: immunocytochemistry, cerebral cortex, muscle, NIH-Image

INTRODUCTION

Hindlimb unloading by tail suspension (HLS) is a non-invasive procedure that simulates some of the effects of weightlessness on antigravity muscles (e.g., soleus atrophy) observed following spaceflight (SF) (Ilyin and Oganov, 1989; Thomason and Booth, 1990; Edgerton and Roy, 1994, for reviews). Although the consequences of unloading have been well-studied in the muscles, little attention has been paid to the possible effects of hindlimb unloading on those areas of the central nervous system related to sensory inputs from muscles.

The primary concern of this experiment was to determine whether γ -aminobutyric acid (GABA) immunoreactivity (GABA-IR) of local circuit cortical neurons could be altered as a result of a non-invasive procedure such as HLS. Our assumption—based on our current behavioral and morphological studies (D'Amelio et al., 1987; D'Amelio and Daunton, 1992; Fox et al., 1993, 1994)—was that muscle atrophy produced by HLS could modify sensory inputs arising from muscle receptors to the cerebral cortex.

We have focused the present report on the behavior of GABAergic neurons since numerous lines of research have demonstrated modifications in the level of GABA-IR or glutamic acid decarboxylase (GAD) immunoreactivity in cortical interneurons when sensory activity is altered by surgical manipulation (Hendry and Jones, 1986; Warren et al., 1989; Akhtar and Land, 1991; see also Jones, 1990).

MATERIALS AND METHODS

Animals

Six Sprague-Dawley rats (200–250 g) were employed for this study. Three served as controls and three were suspended (HLS) by the tail for 14 days.

Suspension Procedure

The suspension procedure (Wronski and Holton, 1987) consisted of the following steps: the tail was cleaned with gauze previously soaked in 70% ethanol, then sprayed with tincture of benzoin for protection

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against adhesive tape irritation, and allowed to dry. A strip of orthopedic tape was attached to a plastic suspension bar and applied to the lateral sides of the tail. The tape was then secured by wrapping a strip of stockette around the tail. The plastic suspension bar was then attached to a pulley system mounted on the top of an acrylic housing unit. In this manner the unloading of the hindlimbs was achieved while the forelimbs were used for locomotion and unimpeded access to food and water. Body weight was recorded daily. Control rats were housed individually in similar cages located in the same room but had no attachments to the tail. The room was maintained at 24°C with a 12-hr light/dark cycle.

Fixation and Sectioning

After 14 days of tail suspension the animals and their controls were deeply anesthetized with Metophane and immediately perfused via the heart with 50 ml 0.9% saline, followed by 500 ml of a fixative made up of 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed the same day, immersed in fresh fixative, and stored at 4°C.

The right hemisphere was coronally blocked between Bregma -1.8 mm and Bregma -3.6 mm, where the somatosensory representation of the hindlimb is conspicuous and associated with the presence of the rostral hippocampus (Paxinos and Watson, 1986). At this level the more rostrally located forelimb representation is no longer present (rostral to Bregma -1.8 the somatosensory cortex contains both hindlimb and the laterally adjacent forelimb representations. The hippocampus is not visible). Coronal sections 40 μ m thick were cut on a Vibratome and collected in TBS (0.05 M Tris buffer-0.9% saline, pH 7.6). Twenty serial sections per animal were used for the staining procedures; 15 were stained for immunocytochemistry, and 5 were Nissl stained with cresyl violet to identify the cytoarchitectonic layers of the hindlimb representation.

GABA Immunocytochemistry

Floating sections were first incubated for 5–10 min at room temperature (RT) with 3% hydrogen peroxide in 10% methanol in TBS and subsequently rinsed 4 times in TBS \times 30 min (RT). The sections were then immersed in GABA antiserum (Chemicon, cat. no. AB131) or control serum (preimmune rabbit serum) diluted at 1:1,000 in TBS for 48–72 hr at 4°C, with orbital agitation. Then they were rinsed 4 times in TBS \times 30 min (RT) and incubated for 60 min (RT) in swine anti-rabbit IgG diluted 1:50 in TBS. The sections were rinsed 4 more times in TBS \times 30 min (RT) and then incubated for 60 min (RT) with rabbit peroxidase-antiperoxidase complex (Sigma) diluted 1:200 in TBS. To develop reaction product the sections were immersed in 12.5 mg diaminoben-

zidine tetrahydrochloride in 50 ml TBS + 5 μ l 30% hydrogen peroxide for 5–8 min. Finally, they were rinsed in TBS, 2 changes \times 10 min (RT), mounted on gelatin coated slides, air-dried, and coverslipped with Permount.

The sections from pairs of experimental and control animals were processed together in the same solutions for consistent immunostaining. For identification purposes, the hemisphere of the control rat was marked with a small hole at the level of the striatum. Sections of each suspended and control pair were placed on the same glass slide for counting of GABA-IR cells.

Methodology for Quantitative Analysis

A Bausch & Lomb inverted microscope equipped with a 25 \times objective was employed to complete the first steps of the analysis. The microscope was set on a table to project the image of the slides at 58 \times magnification.

The hindlimb somatosensory cortex was identified in Nissl-stained slides by the prominent aggregation of granular cells in layer IV. The boundaries of the hindlimb representation were drawn on a piece of white paper. The projected image of the sections stained with GABA antiserum was superimposed on the drawing, and GABA-IR cells intensely or moderately stained were marked on the paper. Blood vessels as well as meningeal foldings served as reference marks for each section. The marking of the cells slightly exceeded the lateral and medial boundaries of the hindlimb representation. Subsequently, the coverslips of the anti-GABA stained slides were removed by soaking in xylene, and the sections were Nissl-stained with cresyl violet and remounted. The Nissl staining of the slides in which the marking of the GABA-IR cells was previously made gave us more confidence in tracing the boundaries of the area and demarcating the cortical laminae based on the prominent granular aggregates of layer IV. The projected image of these sections was drawn on a translucent sheet of paper. The drawing included the boundaries of the hindlimb representation, the reference marks, and the dividing lines of six cortical layers identified as layers I, II/III, IV, Va, Vb, and VI (see Zilles and Wree, 1985). This drawing was then overlaid on the paper that had the markings of GABA-IR cells. The boundaries of the hindlimb cortex were then corrected and GABA-IR cells were counted in each layer on the translucent paper (Figs. 1, 2).

The image of each layer on this translucent sheet was captured into a Macintosh Centris 650 computer using a Sierra Scientific Model MS4030 CCD tube camera that had a macro Nikon/Nikor 55 lens and a Scion Technology LG-3 frame grabber board in the Nubus slot of the computer. Version 1.54 of the public domain NIH-Image image analysis software (written by Wayne Rasband at the U.S. National Institutes of Health) was used

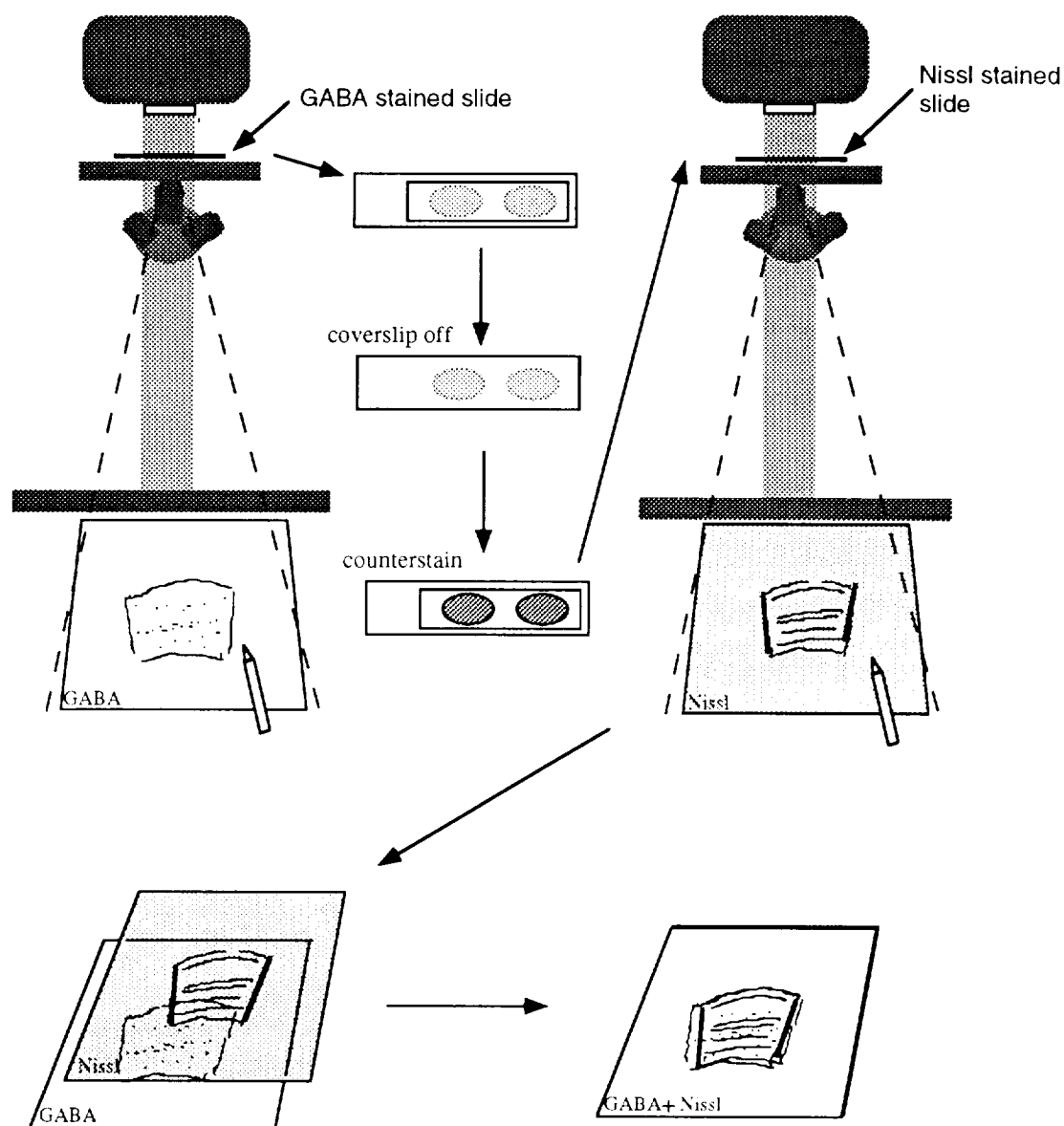


Fig. 1. Schematic drawing of method used for quantitative analysis. See text for details.

for image acquisition and for area measurement of each of the six layers. (The software is available electronically via Internet by anonymous ftp from zipper.nimh.nih.gov or from Library 9, the MacApp forum on CompuServe and on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, Part number PB93-504868.)

An image of standard square inches etched in the copy stand was also captured and then used to compute the correction factor for the distortion of the aspect ratio introduced by the camera lens and the computer monitor. Quantitative measurements of the cortical layers were done blind by one of us (L.C.W.). The digitized images were magnified at $2\times$, and a sharpening filter was used

prior to measuring. Measurements are based on four to eight GABA/Nissl-stained slides for each of the three rats in each group. The measurement data and the number of GABA-IR cells for each layer were entered into Microsoft Excel v.4.0. The frequency of GABA-IR cells/mm² area in each layer for each treatment group was then computed.

RESULTS

The number of GABA-IR cells/mm² of the hind-limb representation was determined for each section lying within the boundary defined by the presence of the

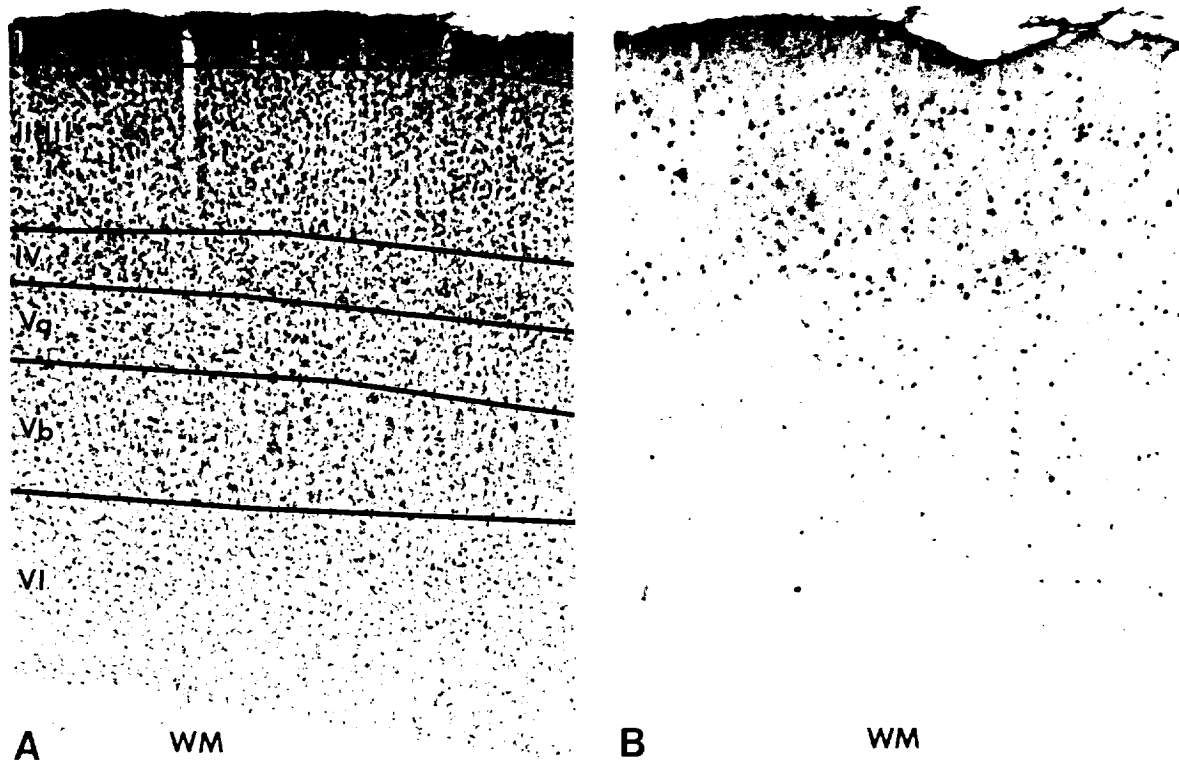


Fig. 2. **A:** Microphotograph of a Nissl-stained coronal section through the hindlimb somatosensory cortex showing the demarcation of the six layers in which counting of GABA-IR cells was done. Note the aggregation of granular cells in layer IV. **B:** Same level as in **A**, stained with GABA antiserum. WM, white matter. Magnification $\times 55$.

rostral hippocampus. A total of more than 7,600 GABA-IR cells were identified. Cell counts were based on four sections for one rat subjected to HLS, on six sections for one control rat, and on eight sections for the four (two HLS and two control) remaining rats. To eliminate differences in staining between pairs of rats, cell counts on sections of HLS and control rats that were processed in the same immunostaining solutions were expressed for HLS as a percentage of control

$$\frac{(\text{HSL GABA-IR cells/mm}^2)}{(\text{CONTROL GABA-IR cells/mm}^2)} \times 100.$$

GABA-IR cells were scattered in all cortical layers, but with the highest concentration in layer IV and lower concentrations in layers I and VI. The number of GABA-IR cells was reduced in rats subjected to HLS. Effects of HLS, expressed as the percentage of reduction in GABA-IR cells, in each cortical layer is shown in Table I. As seen in this table, the reduction in GABA-IR cells varied among cortical layers, with significant reductions occurring in layers Va and Vb.

Although quantitative assessment of GABAergic

terminals ("puncta") targeting pyramidal cell soma and processes was not performed, it was obvious that they were markedly reduced in number in layers Va and Vb when compared with controls (Fig. 3).

DISCUSSION

While it is well documented that the unloading of antigravity muscles by hindlimb suspension leads to atrophy, alterations of neuromuscular units, changes in contractile properties, and the loss of coordination of muscular contraction among different muscle groups (for review, see Edgerton and Roy, 1994), consequences of the unloading at the level of the cerebral cortex have not previously been addressed. Our results indicate that unloading of the hindlimbs results in a significant reduction in immunoreactivity of GABAergic cells and terminals in layers Va and Vb of the rat hindlimb somatosensory cortex.

Several lines of evidence lead us to suggest that the cortical changes reported in this study result from altered proprioceptive inputs from hindlimb muscle receptors without neglecting the possibility of participation of joint

TABLE I. Percentage of GABA-IR Cells in HLS Relative to Control Rats

	Cortical layer					
	I	II/III	IV	Va	Vb	VI
Pair A HLS/C %	108.03	93.56	116.38	65.01	87.35	113.13
Pair B HLS/C %	81.23	111.45	87.48	75.72	81.55	64.60
Pair C HLS/C %	95.72	62.61	57.84	61.02	64.90	47.26
Mean	94.99	89.21	87.23	67.25	77.93	75.00
SD	13.41	24.71	29.27	7.60	11.65	34.14
SEM	7.74	14.27	16.90	4.39	6.73	19.71
% Decrease	5.01	10.79	12.77	32.75	22.07	25.00
<i>t</i> test	0.65	0.76	0.76	7.46	3.28	1.27
<i>P</i> value	>0.10	>0.10	>0.10	<0.01*	<0.05*	>0.10

*Significant difference, by one-tailed test.

receptors and tendon organs. First, although we have previously shown pronounced myofiber atrophy of anti-gravity muscles accompanied by severe alterations in a significant number of motor units immediately after HLS or SF, i.e., degeneration of axon terminals and decrease in the number or absence of synaptic vesicles (D'Amelio et al., 1987; D'Amelio and Dauntun, 1992; for review, see Edgerton and Roy, 1994), no morphological changes in muscle spindles or other sensory structures have been revealed by either light or electron microscopic observations of the same material (D'Amelio, unpublished results). It is therefore likely that after HLS or SF sensory receptors continue to convey signals to the cerebral cortex from "slow" weight-bearing muscles (e.g., soleus), as well as from the predominantly "fast" non-weight-bearing muscles (e.g., tibialis anterior) of the hind limbs.

Second, since receptors of the affected "slow" extensors (e.g., soleus) and the relatively unaffected "fast" extensors (e.g., lateral and medial gastrocnemius) and "fast" flexors (e.g., tibialis anterior) apparently remain operative following HLS or SF, a mismatch of afferent messages from these muscles to the cerebral cortex should be expected. Since in normal conditions stretching of the antigravity soleus muscle evokes heterogenic reflexes in lateral and medial gastrocnemius and tibialis anterior (Nichols, 1989; see also Cope et al., 1994), an imbalance in the reflex responses of these synergetic muscles is most likely responsible for the disruption of gait previously demonstrated by us following HLS and SF (Fox et al., 1993, 1994). That such an imbalance can lead to changes in the cerebral cortex has been demonstrated by Sanes et al. (1992). These investigators have suggested that sensory inputs from muscle receptors are used to adjust the neural circuits related to the specific output functions of the motor cortex and that a mismatch between cortical outputs and sensory inputs during active limb movements (e.g., during walking) can lead to the reorganization of the cortical motor outputs. Such goal-directed reorganization would be designed to

optimize function (e.g., walking) under the conditions of altered inputs from hindlimb muscles.

Thus, the modification of sensory inputs to the central nervous system due to altered functioning of hindlimb muscles, along with the requirements for reprogramming of motor outputs to compensate for the changes in structure and function of those same muscles, could lead to plastic modifications of the circuitry at the cortical level. In these modifications local circuit GABAergic neurons of the cerebral cortex are the most logical candidates to modulate the discharge frequency of pyramidal cells (Jones, 1993) since: 1) the majority of identified local circuit neurons in the cerebral cortex are GABAergic (White, 1989); 2) GABAergic cells are present in all layers of the mammalian cerebral cortex (Ribak, 1978; Houser et al., 1984; White, 1989); and 3) the main synaptic targets of all classes of GABAergic neurons are the pyramidal cells and their processes (White, 1989). Furthermore, experiments primarily concerned with neuronal receptive fields in the somatosensory cortex have shown that GABA-mediated intracortical inhibition specifies size and thresholds of receptive fields of major neuronal subgroups (Hicks and Dykes, 1983; Dykes et al., 1984; see also Jacobs and Donoghue, 1991). It has been shown that the cortical substrate subserving tactile and proprioceptive limb placing—which is deeply disturbed after HLS (Fox, unpublished data)—coincides with a dense subfield of large pyramidal neurons in the deeper part of layer V (De Ryck et al., 1992). In our experiments, layer V showed the most pronounced reduction of GABA-IR cells.

In short, as a result of the selective and differential effects of HLS on weight- and non-weight-bearing muscles, corticospinal fibers would influence motoneuronal pools with either a significant number of abnormal axon terminals innervating the atrophic antigravity muscles or with normal axon terminals innervating non-weight-bearing muscles having minimal or no alterations. As a consequence, disturbances in the afferent signaling and



Fig. 3. Microphotographs of hindlimb somatosensory cortex at the level of layer Vb stained with GABA antiserum. **A:** Tail-suspended animal. The pyramidal cells appear almost totally deprived of peripheral GABA-IR terminals. Note that the neuropil also shows very few terminals (arrowhead) compared with the control in B. **B:** Control animal. Pyramidal cells surrounded by GABA-containing terminals (arrow). Numerous GABA-IR terminals are also conspicuous in the neuropil (arrowheads). PC, pyramidal cell; G, GABAergic cell. Magnification $\times 800$.

feedback information from intramuscular receptors (particularly muscle spindles) to the cerebral cortex would trigger an imbalance in the reflex organization of these synergetic muscle groups. In turn, pyramidal tract neurons processing altered sensory information would respond with changes in the rates of discharge that are modulated by GABAergic neurons.

The emphasis put on muscle spindles over other receptor types as responsible for the changes has a reason, although admittedly speculative. Electrophysiological studies of the rat somatosensory cortex suggest an overlap (co-extension) of sensory and motor areas ("sensorimotor amalgam"), particularly at the level of the hindlimb representation where layer V contains large pyramidal cells that extend over, without interruption, from the motor cortex (Hall and Lindholm, 1974). This type of cortical organization would seem to lend support to the hypothesis first proposed by Phillips (1969) that information from muscle spindles to the cerebral cortex is relayed through an oligosynaptic transcortical spindle circuit for proprioceptive signals whose efferent limb is the corticomotoneuronal projection (for discussion, see Hummelsheim and Wiesendanger, 1985). Several subsequent studies have provided more evidence in favor of this hypothesis (Landgren and Silfvenius, 1969, 1971; McIntyre, 1974; Wiesendanger and Miles, 1982; Matthews, 1991).

Whether the decrease in GABA immunoreactivity is due to alterations in its synthetic activity or depletion due to increased release is a matter of speculation that will require additional studies (e.g., in situ hybridization). Furthermore, electrophysiological recordings will be necessary to assess patterns of activity and receptive field size of cortical neurons influenced by GABA-mediated inhibition under the same conditions. Since the changes we have described are presumably transient [normal gait is recovered after several weeks (Fox et al., 1993, 1994)], it would be important to investigate changes in GABA-IR during the recovery process and to assess whether these alterations may become irreversible given a sufficiently long period of hindlimb unloading.

An essential difference between HLS and sensory deprivation by surgical means is that the former alters GABA-IR in the somatosensory cortex through non-invasive unloading of weight-bearing muscles. For example, Warren et al. (1989) reported a 16% decrease of glutamic acid decarboxylase (GAD) immunoreactive cells in layer IV of the rat hindlimb somatosensory cortex 2 weeks after transection of the sciatic nerve. In experiments conducted in monkey visual cortex after 2–3 weeks of eye enucleation, Hendry and Jones (1986) found a 45% reduction of GABA-IR cells in layer IV. In the same region, these investigators also showed a 36% decrease of GABA-IR cells 11 weeks after eyelid suture.

We believe that HLS generates a more "realistic" chain of events than surgical deafferentation since the afferent input is not interrupted but rather significantly disrupted by the environmental manipulation. Our results suggest that non-invasive manipulations of the neuromuscular system (e.g., hindlimb suspension, alterations in gravitational forces as in spaceflight or hypergravity) can have significant effects on cortical circuitry. Other lines of work based on non-invasive procedures support this possibility, e.g., learning of new movements, compensation of rearranged movements, limb positioning effects (see Jenkins et al., 1990; Merzenich et al., 1990; Sanes et al., 1992). Since severely disruptive surgical interventions are rare but the central nervous system must constantly adjust movements in response to altered environmental conditions, we believe that studies in intact animals should be pursued to help clarify the mechanisms of cortical plasticity and adaptation under natural conditions.

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Damage and Recovery of Otolithic Function following Streptomycin Treatment in the Rat^a

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The toxic action of chronic administration of streptomycin sulfate (STP) on the vestibular hair cells of mammals is well documented.^{1,3-6} Preliminary findings of our group in pigmented rats described severe alterations of motor abilities but an absence of deleterious effects on semicircular canal function (assessed with postrotatory nystagmus) or auditory function (assessed with evoked auditory potentials) after prolonged treatment with STP. These results suggest that STP specifically disrupts otolith organ function in the rat.^{3,9}

Recently, we described gradual recovery of vestibular biochemistry and function in guinea pigs following chronic treatment with STP.⁷ In a morphological study in guinea pigs treated with gentamicin rather than STP, hair cell stereocilia were regenerated after discontinuation of gentamicin injections.²

Because mature rodents are considered to have ceased production of sensory and neuronal elements, these findings are intriguing, and they encouraged us to investigate further the deleterious effects of STP and the possible mechanism involved in recovery after chronic administration of this antibiotic in the mammalian ear using the pigmented, Long-Evans rat as a model. The aim of our work is (a) to confirm an otolith organ toxicity for STP, (b) to identify the cell type affected, and (c) to assess whether recovery occurs in the pigmented rat.

In this paper we report analysis of swimming behavior and morphology by optical microscopy of the sensory epithelium of the utricle in the pigmented rat during and following STP treatment.

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METHODOLOGY

Treatment Protocol

Twenty-day-old male Long-Evans rats were used in this study. Seventeen animals were injected daily intramuscularly for 48 to 57 days with 400 mg/kg body weight of STP (PISA Laboratories, Mexico) dissolved in physiological saline (SPS). Eleven rats served as controls and received SPS injections for the same time interval and conditions as their experimental comates. Three of the 57-day-treated rats and three of the SPS-injected animals were used to follow recovery for 3 to 12 weeks and did not receive any STP or SPS beyond the 48th day.

Swimming Analysis

Swimming behavior was assessed at approximately one-week intervals by placing the rats in a water tank at 27°C and recording, on videotape, swimming activity for 45 sec. Analysis and classification of swimming patterns were performed after the test.

TABLE 1. Percentage of Rats Displaying Each of the Disrupted Swimming Patterns

Experimental Condition	Swimming Characteristics			
	Vertical Swimming with Roll	Barrel Rolling	Corkscrew Swimming	Forward/Backward Looping
48 Days of Treatment	90	60	80	40
3 Weeks Post Treatment	100	0	33	0

Morphology

After completion of each experimental manipulation, two of the 48-day-treated rats and two of the treated and allowed to recover animals, plus two of the SPS injected rats were deeply anesthetized and transcardially perfused with aldehyde fixative. The auditory bullae were extracted and postfixed in 1% osmium tetroxide, dehydrated, and embedded in Araldite. Vestibular organs and half turns of cochlear duct were sectioned at 1- μ m thickness, stained with methylene blue and azure II, and examined by brightfield microscopy.

RESULTS AND DISCUSSION

Abnormal swimming patterns consisting of vertical swimming with rolls, barrel rolling, corkscrew swimming, and forward and backward looping were observed with varying frequencies in rats treated with STP. None of these responses was observed in any test of control rats. Eight weeks post treatment, vertical swimming with rolls remained in all rats. One of the three rats showed corkscrew swimming, but no rat showed barrel rolling or looping. Hence, partial functional recovery was observed (see TABLE 1).

Histological examination of STP-treated rats revealed that in the utricular macula sensory cells presented fused stereocilia and pyknotic nuclei. In addition, some of these sensory cells were in the process of being extruded from the epithelium



FIGURE 4. One micron sections of utricle maculae cut perpendicular to endolymphatic surface. (A) Control (saline injected). Hair cell bodies are closely packed together, and stereocilia bundles 10-15 cm at regular intervals above epithelial surface. Some stereocilia are broad (thick arrows) and some hair cell nuclei are pyknotic (small, thin arrows). (B) Streptomycin treated and "recovered". Sensory epithelium appears more uniform than in B but slightly atrophic. Hair cells have normal appearance, stereocilia are not broad, but density of bundles is reduced (arrows). O, otocyst; G, gelatinous layer of otolith membrane; J, type I hair cell; B, basal lamina; SC, supporting cells; MF, myelinated nerve fibers.

(FIG. 1). In contrast, sections of the cristae and organ of Corti appeared normal. In STP-treated and "recovered" animals, neither fused macular hair cell stereocilia nor pyknotic nuclei were observed, but bundle density was reduced. Thus, a partial recovery of sensory epithelium morphology also occurred.

The abnormal swimming behavior observed in rats chronically treated with STP is identical to that observed in congenitally otolith-deficient mice and supports our postulation of otolith organ-specific toxicity of STP in the rat. This is confirmed by our observation that degeneration of hair cells is restricted to the macular organs of antibiotic-treated rats. The partial reversibility of abnormal swimming behavior in animals eight weeks following treatment is in accord with our observations of partial morphological recovery in the same animals. These results show that hair cell and functional recovery can occur in a mammal subjected to prolonged treatment with a clinically relevant toxic agent.

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Effects of spaceflight and hindlimb suspension on the posture and gait of rats

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Introduction

Instability of posture and gait in astronauts following spaceflight (SF) is thought to result from muscle atrophy and from changes in sensory-motor integration in the CNS that occur during adaptation to micro-G. Individuals are thought to have developed, during SF, adaptive changes for the processing of proprioceptive, vestibular and visual sensory inputs [1] with reduced weighting of gravity-based signals and increased weighting of visual and tactile cues [2]. This sensory-motor "rearrangement" in the CNS apparently occurs to optimize neuromuscular system function for effective movement and postural control in micro-G. However, these adaptive changes are inappropriate for the 1g environment and lead to disruptions in posture and gait on return to Earth.

Few reports are available on the effects of SF on the motor behavior of animals. Rats studied following 18.5-19.5 days of SF in the COSMOS program were described as being "...inert, apathetic, slow" and generally unstable [3, p. 334]. The hindlimbs of these rats were "...thrust out from the body with fingers pulled apart and the shin unnaturally pronated" [3, p. 335]. On the 6th postflight day motor behavior was described as similar to that observed in preflight observations.

Improved understanding of the mechanisms leading to these changes can be obtained in animal models through detailed analysis of neural and molecular mechanisms related to gait. To begin this process the posture and gait of rats were examined following exposure to either SF or hindlimb suspension (HLS), and during recovery from these conditions.

Methods

Subjects

Eighteen Sprague-Dawley rats (130 to 155 g) were obtained from Harlan Laboratories for the SF study. For the HLS study 20 Sprague-Dawley rats (230 to 255 g) were obtained from Simonsen Laboratories. Rats were randomly assigned to groups and were maintained on 12:12 hr light:dark cycle with food and water available *ad libitum* throughout the experiments.

Procedures

SF Conditions. During the 14 day flight on STS-58, rats (n=6) in the Flight (FL) condition were maintained individually in

cages measuring 10.2 X 10.2 X 20.3 cm. Rats (n=6) in the Flight Control (FC) condition were housed in cages of the same size in the ground colony room. Vivarium Control (VC) rats (n=6) were housed in standard rat colony cages modified by placing a clear acrylic divider lengthwise to create two sections measuring 22.9 X 45.7 X 20.3 cm. VC rats could rear during the 14 days of the flight while those in the FC and FL conditions could not. Animals in the VC and FC conditions were placed into appropriate cages on the first day following launch. All animals were transferred from the cages used during the flight period into metabolic cages (30.5 X 30.5 X 30.5 cm) between 6 and 7 hr after the time of landing (i.e., immediately after the initial test).

HLS Conditions. HLS was accomplished with a modified version of the Morey-Holton technique [4]. A device was constructed using Fas-Trac to attach a connector to the rat's tail. This connector then was attached to a swivel hook that allowed free, 360° rotation within the 30.5 X 30.5 X 30.5 cm cage. The height of this hook was adjusted so the hindlimbs of the rats were just off the floor when in full extension. With this procedure the hindlimbs of the rats (n=10) were "unloaded" (HLU) from postural (anti-gravity) support, and the animals moved about by propelling themselves with the forelimbs. Rats (n=10) in the control group (HLC) lived in similar cages but were not attached to the suspension system.

Testing Procedures. Beginning 6 hr after landing SF rats were encouraged to locomote across a walkway (15 X 30 X 150 cm) with clear acrylic walls and a glass floor. Light was passed through the glass from the front to the back edge so that foot contact could be viewed from below and recorded on videotape [5]. This video record was combined with a profile view of the rats on a split-screen display. Following the initial test on the day of landing each rat was tested after 2, 4, 7 and 14 days of recovery (Days R2, R4, etc.). Quantitative assessments of posture and limb movements were made by determining X-Y coordinates of identifiable points using a PEAK™ Technologies Motion Analysis system.

In the HLS study rats were tested within 5 min following removal from the suspension device (R0) and then on R2, R7, and R14. (In addition, these animals were exposed to 3-5 tests of the air-righting reflex and a 45-s swim test prior to testing for gait on each test day.)

Results

Posture

When first tested 6 hr. after return from SF (R0) FL rats walked slowly with the back dorsiflexed, the hindquarters lower than in FC and VC animals and with the tail dragging on the floor. Limb movements of FL rats could be described as "hesitant." Immediately after removal from HLS the rats also walked slowly, but HLU rats walked with the back straight or ventro-flexed, the hindquarters higher than HLC rats and with the tail held off the floor. Both FL and HLU rats walked with a sinusoidal, vertical oscillation of the pelvic region.

Foot Placement and Hindlimb Extension during Walking

On R0 SF rats walked with extreme dorsiflexion of the ankle (plantar extension) producing atypical foot placement that resembled that seen in 10-day old rats [6] in which the foot pads contact the floor only at the end of the stance phase. In contrast, all HLS rats walked with normal foot-pad contact.

The elevation of the hindquarters and extension of the hindlimb observed in the assessment of posture were examined further by evaluating the distances from the base of the tail to the floor (Fig. 1A) and to the foot (Fig. 1B) respectively during walking. The base of the tail was significantly closer to the floor on R0 in FL ($p < .01$) than in FC or VC rats. On R2 the base of the tail of FL rats was higher than in FC or VC rats ($p < .01$), but was not different on R7 or R14. In contrast, the base of the tail was significantly higher off the floor in HLU animals than in control rats on R0 through R7 ($ps < .001$), but not different from control rats on R14. The hindlimbs were more flexed in FL than in FC or VC rats on R0 ($p < .005$) but more extended on R2 through R7 ($ps < .05$). The hindlimb was more extended in HLU rats than in control rats on R0 through R7 ($ps < .03$), but not different from control rats on R14.

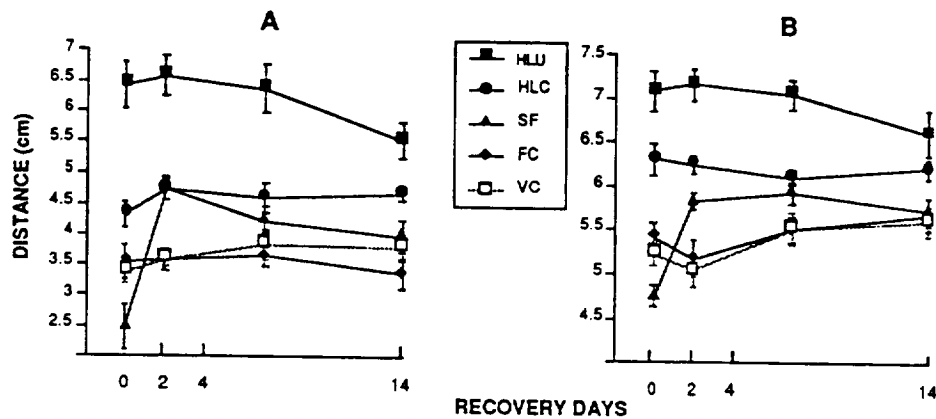


Figure 1. Elevation of the hindquarters measured as distance from the base of the tail to the surface of the walkway (Panel A) and leg extension measures as distance from the base of the tail to the foot (Panel B)

Discussion

The hindlimb extension, dorsiflexion of the ankle, and vertical oscillation of the pelvic region observed in FL and HLU rats may result from an altered balance of flexor-extensor muscles that is produced by treatments which decrease the "mechanical use" of weight-bearing muscles. Atrophic effects in SF and HLS rats are muscle-specific with slow extensors most affected, fast extensors moderately affected and flexors least affected [7, 8]. The effects of SF on physiological properties

of muscle are less well known, but documented protein changes in muscle are associated with lower excitability of the extensor pool while the flexor pool is unaffected. Assessments of muscle function in Salyut crewmembers indicated decreased strength and an increased ratio between maximum amplitude of EMG and muscle torque in leg extensors with no change in flexors [9]. These changes presumably result in a shift toward relative dominance of flexors over extensors.

Flexor dominance could produce dorsiflexion of the ankle during stationary stance as reported here. In addition, when there is extreme atrophy of the soleus, the relatively less compromised biarticular gastrocnemius may become increasingly important in dynamic ankle extension. Compromised activity of the soleus could contribute to poor adjustment of the foot prior to touchdown and to dorsiflexion during early stance when activity of fast extensors normally is minimal. Because maximal force of the gastrocnemius is length-dependent, gastrocnemius activity that contributes to ankle extension may vary as the length of the muscle changes due to biomechanical factors related to knee and ankle extension. Such changes in force could produce the vertical oscillation of the pelvis observed here. Hyper-extension of the leg in SF and HLU rats may be a postural adjustment to facilitate ankle movement by adjusting gastrocnemius length to produce proper force for adaptive ankle extension.

Footnotes

Both experiments conformed to the Center's requirements for the care and use of animals. Support was provided by NASA Grant NCC 2-723 and SJSU Foundation Grant 34-1614-0071 to R.A. Fox.

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